ENVIRONMENTAL EFFECTS ON DEVELOPING BIRDS: SHORT-TERM ADJUSTMENTS WITH LIFE-LONG IMPACTS

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ENVIRONMENTAL EFFECTS ON DEVELOPING BIRDS: SHORT-TERM ADJUSTMENTS WITH LIFE-LONG IMPACTS

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ABSTRACT

Our understanding of the linkages between developmental environment and emergent phenotypes in non-avian reptiles has grown immensely since the discovery of temperature-based sex determination half a century ago, yet little is known how developmental temperature affects avian phenotypes despite their shared evolutionary history. In birds, the sum of knowledge on this topic is restricted to just two precocial families, Megapodidae and Anatidae, where data suggests developmental temperature can have significant effects on avian phenotypes, with important implications for future survival and fitness. In these four chapters, I provide the theoretical justification for future studies on developmental plasticity based on previous experiments and provide an analytical framework for study. In addition, I use both experimental and long-term datasets to analyze the effects of costly developmental environments in a model Passerine bird, the Tree Swallow (*Tachycineta bicolor*). Emerging research is beginning to illustrate the developmental environment in birds has the potential to expand our understanding of what drives fitness, survival, and the evolution of life history traits in birds.

BIOGRAPHICAL SKETCH

Born and raised in the cross timbers region of Central Oklahoma, Jeremy Ryan Shipley (henceforth known as J. Ryan Shipley, thanks to his parents giving him a first name but calling him by the second, endlessly confusing the Cornell administration and his advisor) … Ryan spent his childhood as most do when growing up in rural America, the mornings were spent working in the general store whereas the hot idyll afternoons were spent under the shade of oak trees, passing the time away with a cold Sarsaparilla. Children were always brainstorming ways to save enough money to finally buy a Redbone Coonhound of their own.

Ryan began his adult academic career at the University of Oklahoma, and after a brief 4-year stint as a budding Architecture student, found himself at odds with his career direction and his lifelong passion, the diversity of life on this planet. At this juncture, Ryan unenrolled himself from the architecture program (without a signed permission slip from his parents) and promptly started taking biology and chemistry course at his first alma mater, The University of Central Oklahoma. It was here that he received his B.S in Biology in 2008 – but was still a boat without a rudder, an explorer without a map and sextant, an undergrad without an internet connection. Ryan knew he wanted a career in academic research but didn't really know where to begin.

It was his return to the University of Oklahoma, where Ryan got his first experience doing independent research (read: unpublishable). A single email asking about the use of stable isotopes to study the trophic ecology of bats would change everything, prompting a meeting with his future M.S. advisor Dr. Jeff Kelly. It was during this 2 ½ year span that Ryan studied seasonal resource use in tropical bat communities, weather radar applications for ecology, honed his programming skills in C and Python, and began his interest in embedded systems engineering.

In the fall of 2012, Ryan began his Ph. D. in Ecology and Evolutionary biology

under the tutelage of Dr. David Winkler (henceforth known as Wink), bird guru. Acceptance into the lab required bringing an undamaged down feather from a Nepalese House Martin to prove your scientific mettle and dedication. After completing this nigh impossible task, Ryan and Wink became close friends, and at Cornell, Ryan was able to pursue his research interests in depth. During this time, Ryan was awarded a Sage-Colman Diversity Fellowship, funding from the Cornell Lab of Ornithology from Richard and Mary Jane Schnoor and Ivy Graduate Research Fellowships, research specific funding from the Graduate School and Department of Ecology and Evolutionary Biology. As a graduate student, Ryan also had the privilege of mentoring 3 undergraduate students; Daniel Gu, Eric Gulson, and Andrew Dreelin in their senior theses as well as Francois Mertil in his Master of Engineering.

Research projects in his M.S. and Ph. D. provided Ryan with innumerable opportunities to slake wanderlust. Dissertation research took Ryan to the jungles of Sabah on the emerald labyrinth of Borneo (not for long, Palm Oil), to the highlands of Mexico exploring volcanic caves for bat species (not for long, lava), to the aquamarine cenotes of Belize (not for long, coastal resort hotel development), to the seemingly infinite sandy deserts (no shortage of sand), flammable *Spinifex* spp. plains, and lush drop-bear laden forests of Australia. It was here in Australia, that he and his wife Lily fostered a lifelong love for the amazing diversity of fuzzy proto-mammals. A particularly memorable research trip was in the Pacific Northwest, where he was hitchhiking through Oregon and the local law enforcement started harassing him. Next thing you know, Ryan had a whole army of cops chasing him through the woods (wait… that was Rambo: First Blood).

As I stir through the remnants of my final latte and the lamp oil runs low, this is where the awkward third person self-aggrandizing narrative ends, and where the actual dissertation begins…

To Lily, the best collaborator and partner anyone could ever wish for.

ACKNOWLEDGMENTS

First of all, I'd like to thank all of my parents, both biological; Tina Murray and Ron Shipley and through marriage; Dennis Murray and Linda Batchelor, for their unwavering support of my education and my passionate pursuit of biology. They never failed to provide encouragement during times of difficulty and always wanted to only see me do my best. I'd also like to thank my grandparents, Beverley Hoehn and Johnnie and Monte Shipley, for instilling at a young age a love for reading and nature. One of my most vivid childhood memories is being read a vintage National Geographic, where the images stirred the curiosity and imagination of a 6-year-old, in that moment is where this story began.

Second, I want to thank my wife Lily for being the most amazing co-conspirator I could ever ask for. In both our professional and personal lives, Lily is exceptionally hard working, infinitely fair-minded, and most importantly, a fierce, fierce friend. She showed me that raw talent isn't enough, self-discipline and hard work are essential for anyone to achieve their potential. Throughout both of our Ph. D.s, Lily has always been supportive of pursuing new ideas and provided an inexhaustible amount of encouragement even when I couldn't see the purpose anymore. Most importantly, I've never met a more genuine person and I feel immeasurably fortunate to have married my best friend and confidant. I truly believe I couldn't have done this without you.

I next want to thank my Ph. D. advisor and dear friend, David Winkler. Throughout my tenure at Cornell, you encouraged me to pursue my interests and offered invaluable advocacy for my ideas. As your student, you provided me the tools to become a better writer and express my ideas more clearly – albeit with some initial

resistance from my end. In addition, without your willingness to share your encyclopedic knowledge of avian ecology and evolution, and encouragement for others to share in your enthusiasm, this dissertation would not have been possible. Finally, on a personal level you've been an irreplaceable mentor and friend, and perhaps most importantly, helped me maintain perspective that straight unbroken paths only exist in geometry and are not part of Nature or Life.

I'd also like to thank my two committee members, Jeremy Searle and Ned Place. Both Jeremy and Ned provided constructive criticism that was instrumental to each individual project and pushed me to consider different perspectives or possible outcomes, ultimately improving the quality of my work. In addition, I'm appreciative of the support I received when I changed the focus of my dissertation to a subject I found more interesting.

My M.S. advisor at the University of Oklahoma, Jeff Kelly, also warrants special recognition. Jeff believed in my potential during a time I was struggling to find direction and offered me an opportunity. Jeff is an incredible mentor and always provided insight and guidance when it was needed the most.

My labmates, both new and old, also deserve recognition and were key to my success in graduate school. Amos Belmaker, Nate Senner, Anna Forsman, and Kelly Hallinger were integral to making me feel welcome in the Winkler Lab during my first year and providing supportive criticism of my research plans at Cornell. My E240 cellmate, Justin Proctor, joined the lab the same time as me and we became close friends almost immediately. Justin is always a source of positivity and encouragement, and I am sure we will remain lifelong friends. I'd also like to thank the two more recent arrivals to

the Winkler lab, Jenny Uehling and David Chang Van Oordt. You both are amazing individuals and seeing your research develop and mature is immensely rewarding. My only regret is we only got 2 years of overlap at Cornell together.

Finally, I'd like to thank all the friends I've made along the way at Cornell. Moving 1500 miles away from Oklahoma after spending the first thirty years of my life there was incredibly challenging, and you made Ithaca feel like home. I'd especially like to thank the 2012 EEB cohort, for making our first year so memorable. I'd also like to thank Jake Berv, Collin Edwards, Erin Larson, Keeley MacNeill, Vegard Saetre, and Jacob Tyrell, for participating in extracurricular activities ranging from Dungeons and Dragons to mountain biking to rock climbing to home remodeling.

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PREFACE

This dissertation is divided into four parts that illustrate how the environment, both during early life and beyond into adulthood – have the potential to shape the phenotype and modulate the expression of traits that ultimately affect survival and fitness. Chapter I provides an in-depth review of what we know about developmental plasticity in birds and provides evidence that both the timing of receiving a cue and the mechanism driving the associated phenotypic response are relevant for our understanding of developmental effects. In addition, it introduces a framework for our understanding of how during early life - the maturity of tissues, their maximal rate of growth, and their potential phenotypic plasticity are interrelated. Chapter II demonstrates that different developmental environments have associated metabolic costs, and this translates to varying resources being allocated to growth in developing offspring. These results illustrate that without additional parental effort, the developmental environment can elicit different phenotypes, albeit in a controlled lab experiment. Chapter III provides evidence for phenotypic mismatch, that a specific developmental phenotype has varying fitness in different environment, with consequences for fitness and/or survival. The analysis shows that Tree Swallows developing at high temperatures have reduced thermogenic capacity via reduced pectoral muscle mass, resulting in hypothermia and death when facing low temperatures. Chapter IV delves into feather morphology, and investigates the function of an underappreciated attribute, the afterfeather. Phenotypic traits that provide multiple functions such as feathers are likely under complex selection, functioning for sexual selection, crypsis, waterproofing, aerodynamics, insulation, and more. However, due to its location– we provide evidence the afterfeather's main function is insulation, simplifying selection across a wide range of climates in Australia.

Chapter 1

Temperature-driven Developmental Plasticity in Birds across the Altricial-Precocial Spectrum

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Abstract

Understanding how early life conditions affect the formation of adult phenotypes is a long-standing question in evolutionary biology. Cues and inputs from the local environment have the potential to initiate alternate developmental trajectories and result in concomitant changes in fitness as adults. Here, we discuss the underlying theory, mechanisms, and selective pressures in the context of the altricial-precocial spectrum in birds and their varying developmental maturity. Developmental plasticity has been overlooked in altricial birds, despite evidence for it in their precocial counterparts, and we suggest the possibility that rapidly developing tissues hatching at an immature state may be prime candidates for adaptive adjustments through developmental plasticity. The distinction between informational and somatic responses to environmental changes helps illuminate such plasticity.

1. Introduction

Parents have a tremendous influence over the environment their offspring experience during early life and this influence is most pronounced in species with extended parental care. In birds, part of the parental influence includes the decisions or limitations of when and where to nest, which profoundly affect the environment offspring will experience during early life. Accordingly, parents decide which strategies to use based on both internal and external cues (Winkler et al. 2014) that provide information about the reproductive environment, enabling parents to maximize offspring fitness and success. Natural selection has favored the use of cues that provide reliable, predictive information about environmental conditions conducive to the production of viable, fit offspring and maximizing individual fitness (Åkesson et al. 2017, Winkler et al. 2014, McNamara et al. 2011). Despite past selection for cue quality in a population, no cues, associated responses, or strategies are perfect, and offspring are exposed to varying temperature, caloric intake, stress, and other factors that impact growth during early life and development (Both et al. 2009). There is a tremendous wealth of studies documenting the allocations that birds make while providing parental care. Here, we develop a complement to that approach, concentrating on chicks as developing organisms, not their parents as mediators of development. ***Specifically, we explore the interactions between constraints and strategic options from the perspective of the developing chick, including both pre- and post-hatch constraints, that can impact chick developmental trajectories and resulting adult phenotypes.***

Understanding how early life conditions, both during incubation and post-hatch, affect developing chicks is relevant for several key reasons (Monaghan 2008). First, the earlier in development an organism receives information about the state of

the environment, the more potential time there is to elicit an appropriate response. During early life, developmental plasticity provides a mechanism for adjusting physiological traits to meet current demands in chicks, while potentially providing a buffering capacity in case of the unexpected (Piersma and Van Gils 2011, Piersma and Drent 2003). Second, continued exposure to suboptimal conditions during development has the potential to create negative consequences later in life, including reduced fitness, body condition, and even lifespan (Monaghan et al. 2012). Third, developmentally plastic traits that are molded in early life have the potential to drive the characteristics and evolutionary trajectory of populations, by modulating the traits of adults and their lifetime reproductive fitness (Ricklefs and Wikelski 2002). Finally, different species and/or populations may have been under vastly different selective regimes for phenotypically plastic responses during development and accordingly, the presentation of a novel cue may result in adaptive, neutral, or even maladaptive plasticity and negative life history consequences may limit the ability to even elicit a response.

Despite the interest in phenotypic plasticity and the adjustment of traits to cope with environmental variation, most of our evidence in birds comes from the analysis of adult phenotypes. Far fewer studies have focused on the developing young before they reach maturity, and of those, most have concentrated on precocial species. Despite the evidence for fitness consequences of variation in the developmental environment in precocial species, far fewer studies have investigated this phenomenon in altricial species (but see recent work by (Andreasson, Nord, and Nilsson 2018, Lodjak et al. 2018, Lodjak et al. 2017, Lodjak et al. 2014). This is unfortunate as altricial birds comprise over 70% of all bird species and, as we will see, both precocial and altricial species go through a similar set of development stages as they mature and

gear up for independence.

Here we summarize and review the available literature on environmental influences on development, concentrating on the effects of temperature, and we review documented fitness effects in both precocial and altricial birds. The relative maturity of different physiological systems at hatch varies in chicks across the altricial-precocial spectrum (Figure 1.1). We provide evidence to advance the idea that physiological maturity of tissues, not their absolute age relative to hatch date, may dictate the potential of a developing tissue to effectively respond to suboptimal developmental conditions. Finally, we conclude with priorities for future research in this field and its potential contributions to the study of both ecology and evolution.

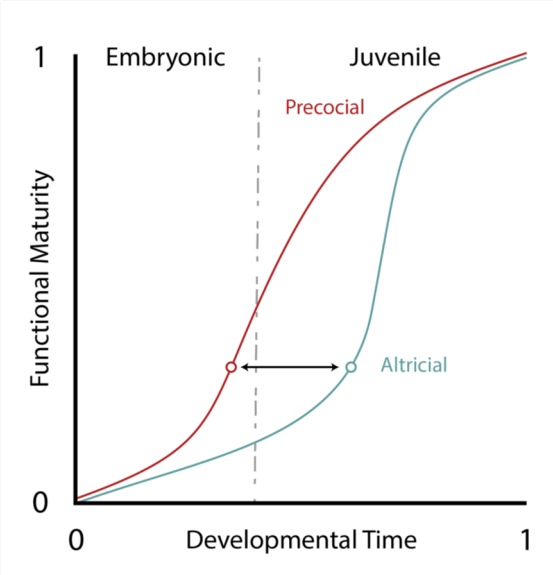

Figure 1.1. Functional maturity and the timing of development. The rate of maturation and growth in altricial and precocial birds start out at a similar parallel trajectory but diverge in late development (dashed line approximates hatching). Consequently, precocial birds hatch at a greater degree of functional maturity in many tissues. If functional maturity underlies the scope of potential developmental phenotypic adjustments, this likely occurs at a later in developmental time in altricial species.

2. Development along the altricial-precocial spectrum

a. Early life and the initiation of developmental trajectories

The environment that organisms experience during cellular differentiation and early tissue development is thought to be key to determining developmental trajectories. Early in life, epigenetic changes via gene promotion and repression are established (West-Eberhard 2003), alternate routes of developmental programming are set into motion (Waddington 1957), and cells undergo rapid growth and differentiation as they advance to their mature state. The "epiphenotype hypothesis" suggests that there is a relationship between the degree or extent of plasticity and when a cue occurs during development. The epiphenotype hypothesis has empirical support (Snell-Rood 2012, Snell-Rood, Swanson, and Young 2015), and there is one key aspect that we find especially relevant: that the relative developmental age of tissues is inversely related to their future developmental plasticity. As we shall see, this potentially greater flexibility for affecting the eventual physiologically mature adult systems may be an unrecognized advantage for the altricial pattern of growth.

b. Timing of development in the avian embryo

From the moment of fertilization to the point of hatching, all birds, regardless of the degree of independence at hatching, go through a similar sequence of 42-46 developmental steps (Starck and Ricklefs 1998). Time spent in the initial 33 stages is similar between bird species, with little difference across the altricial-precocial spectrum and between distantly related families (Starck 1998). Analyses of growth rates and developmental sequences suggest that embryonic growth trajectories are parallel during early stages regardless of the extent of precociality, and only diverge in late embryogenesis (after ca. stage 37, Figure 1.1)(Starck 1998). The early stages of

avian development establish the basic functional systems for life: cells begin to differentiate and organize into tissues, organs begin to form, and individual physiological systems (skeleto-muscular, sensory/nervous, digestive/excretory) begin their paths along their trajectories towards maturity. Even though the basic organization into organ system precursors is shared by very young birds of all species, the pace of differentiation and development of mature physiological function varies from species to species according to the ecological demands that each of them face as developing birds. Thus, sensory and locomotory development is quite varied, and the only real constant across all birds is the early development of digestive and circulatory systems that fuel growth. Given these differences in developmental maturity at hatch, it was previously suggested that the difference along the altricial-precocial spectrum lies in where hatch date occurs along the 42 developmental steps, that altricial birds simply hatch at an earlier developmental stage than do their precocial counterparts (Portmann 1955).

c. Comparison of tissue development along the altricial-precocial spectrum

The developmental stages of avian embryos delineate the origins of all organ systems. However, the existence of a precursor and the initial differentiation and proliferation of organ cells is not equivalent to their functional maturity. Chicks across the spectrum hatch with tissues at vastly different degrees of functional maturity. Tissues that play essential roles during early stages of development (e.g., heart or components of the digestive tract) tend to be at similar states of maturity across the altricial-precocial spectrum, whereas tissues associated with behavior outside the nest and independence (e.g., flight that requires pectoralis muscles and feathers) are likely to develop at a later stage (Starck 1998).

Altricial species hatch with most tissues at a functionally immature state: altricial chicks have poor thermoregulatory, sensory and cognitive capabilities and require considerable parental care (Starck 1998). In contrast, precocial chicks hatch with some fully functional skeletal musculature, typically the leg muscles involved in locomotion, possess down feathers, are alert, and begin thermoregulation shortly after hatch (Starck 1998). Thus, when we view avian tissue development through the lens of functional maturity rather than in terms of differentiation or organizational steps, Portmann's (1955) characterization can be amended: altricial species at hatch tend to have more tissues at an earlier stage of functional maturity than do comparable precocial species. The distinction between altricial and precocial species lies in the functional maturity of the tissues at hatch, not in the developmental stage attained.

A trade-off exists between the intrinsic rate of growth and the functional maturity of a given tissue (Ricklefs, Shea, and Choi 1994). Considerable evidence for this exists in birds, where altricial species born at an immature state grow three to four times faster than their precocial relatives hatching with more functionally mature tissues (Figure 1.1) (Ricklefs 1979). The growth rate/functional maturity trade-off has been explored in several tissues, including striated skeletal muscle, bone, and nervous tissues. For example, in skeletal muscle the daughters of myoblast cells incorporated into muscle fibers can no longer proliferate, thereby limiting the growth potential of the muscle (Moss and Leblond 1971; Dayton and Hathaway 1991). Functional maturity in skeletal muscle is associated with reduced water content, indicative of the increase in the number of myofibrils, mitochondria, myoglobin, and capillary density in the mature, functioning muscle cell. Long bones of terrestrial vertebrates have cartilaginous growth zones whose size determines both the rate of elongation (embryonic function) and, inversely, the mechanical strength of the bone (mature

function) (Kirkwood et al. 1989; Carrier and Leon 1990; Pines and Hurwitz 1991; Swartz et al. 1992). Rapidly growing cartilaginous zones in bones have lower mechanical strength than adult bones that are no longer growing (Rath et al. 2000). With neural tissue it is the same: differentiated, functioning neurons cannot, at the same time, proliferate.

Viewing this growth rate/maturity trade-off together with the flexibility/maturity trade-off from the epiphenotype hypothesis, we reason that altricial birds, with their preponderance of fast growing functionally immature tissues are likely more developmentally plastic than their precocial counterparts. If true, this would suggest that environmental cues received in the post-hatch nesting environment would have the strongest effects on altricial species. The altricial bird has typically a smaller body size and associated smaller energetic reserves, shorter nesting period, and faster growth rates than their precocial counterparts, meaning that stochastic downturns in food availability which are likely to occur during development will comprise a greater proportion of the total nesting period.

3. Mechanisms underlying adjustment during development

a. Informational and somatic responses

Determining how organisms respond to environmental variation during development and whether phenotypic responses are adaptive is key to understanding the evolution and maintenance of developmental plasticity. An important distinction is whether adaptive changes in growth arise from responses to environmental cues to initiate alternate developmental trajectories (informational response) or reflect the action of environmentally imposed constraints or use (somatic response) ((Nettle and Bateson 2015) Schew and Ricklefs 1998, Smith-Gill 1983). Responses to both cues

and shortfalls in resources can be tuned by selection, and informational and somatic responses both have strong roles to play in molding adult phenotypes during development (Figure 1.2).

Informational responses rely on response to a developmental cue to provide relevant information about future conditions (Nettle and Bateson 2015). This type of plasticity is thought to evolve when there is a strong and reliable correlation between the cue and the future environment. Provided this correlation, natural selection has

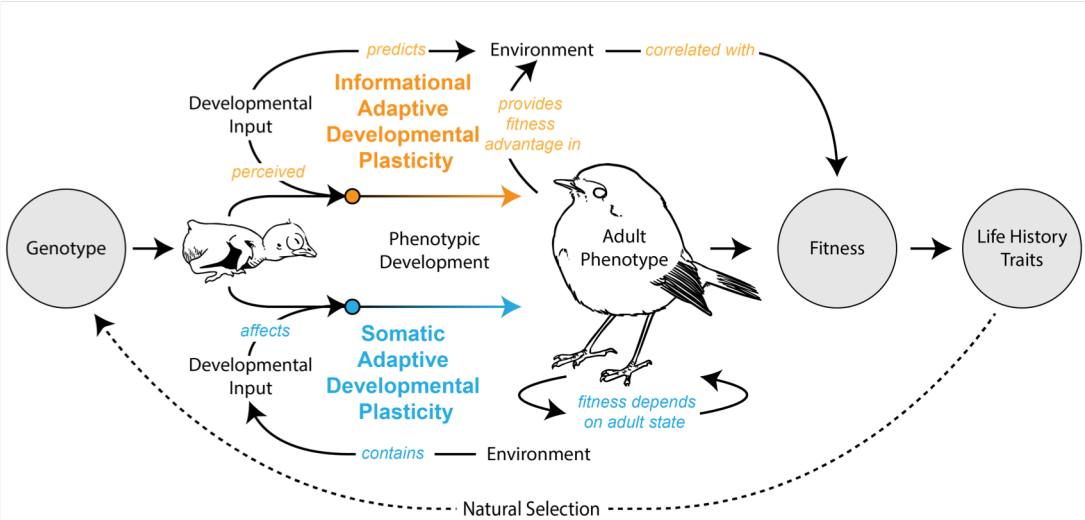

Figure 1.2. Comparison of informational and somatic adaptive phenotypic responses – The effects of early life development is connected to adult fitness and ultimately demography and selection for life history traits. In informational adaptive developmental plasticity, an input perceived by a developing individual is correlated with future conditions and natural selection filters phenotypic responses that increase fitness in the adult phenotype. In contrast, somatic adaptive developmental plasticity is driven by inputs during development that affect phenotypic expression in a causal manner and the fitness benefits depends on the state of the adult individual.

been able to filter through the possible responses to different cue values, associating best-adapted responses with each.

In somatic responses, the relationship between the developmental input and the phenotype are causal, and it does not depend on the correlation between an environmental cue and fitness in a future environment. In this sense, the resultant phenotype is a product only of developmental circumstances and the internal somatic state; and it occurs when alternatives are unlikely to evolve because of binding physical constraint (e.g., temperatures too low for any reallocation of energy budget to accommodate, caloric intake below that needed for maintenance, let alone growth). Differences in fitness based on somatic responses are likely context-dependent; the benefits of a specific phenotype will depend on the state of the adult individual. Developmental inputs such as early life nutrition, parental provisioning, and thermal history are likely to have pervasive impacts that determine overall function and survival for the rest of the lifetime. Nettle and Bateson (2015) note that somatic responses historically have been ignored, despite the tenet in behavioral ecology that animal decisions should be state-based.

In birds, somatic responses are thought to usually entail limitations of growth from environmental conditions, including reduced temperatures or parental provisioning (Schew 1995), a "making the best of a bad situation" response. In addition, there is the potential for somatic responses to be induced by use or demand, such as muscular hypertrophy or atrophy of the digestive tract during periods of feast or famine. In contrast, informational responses can include differential allocation to tissue growth at the expense of others (Dissertation Chapter 2, (Killpack and Karasov 2012), preferential routing of nutrients to storage before migration or breeding

(Piersma and Jukema 2002), or delaying the onset of maturity (Emlen et al. 1991) in situations where the growing organism has multiple developmental trajectories available.

For species that are relatively inflexible in their growth trajectories, somatic reductions in growth rate are likely to result in stunting and reduced fitness unless there is compensatory growth later in development (Metcalfe and Monaghan 2001). However, evidence confirms that there are limits to the catch-up rate, and compensatory growth, with its faster-than-normal development, has costs in reduced functionality across a diverse range of vertebrates (Hector and Nakagawa 2012, Criscuolo et al. 2011, Alonso-Alvarez et al. 2007). In other words, phenotypic traits that may promote offspring survival in poor conditions are likely to come with a cost that is incurred late in life (Monaghan et al. 2012). Alternatively, if growth rates are inherently more flexible, delaying maturation requires only additional time, and not compensatory growth and the associated costs to reach adult size. However, there are selective limitations from predation risk while in the nest, and delaying maturity may come with other indirect costs that select against delay (Conway and Martin 2000b).

b. Selected strategic responses to external information or changes caused by use?

The organization and development of many organismal traits are driven by shared genetic programs, upon which selection acts. For example, despite the amazing diversity of beak shapes in birds, the dynamics of growth appear to be governed by differences in expression of a few underlying genetic loci (Lamichhaney et al. 2016, Fritz et al. 2014). Despite this trajectory set by early life programming, development of the beak is also guided via behavioral responses, providing another mechanism to respond to challenges from the environment. For example, variation in Oystercatcher

(Haematopodidae) bill morphology is related directly and reversibly to the hardness and size of prey recently processed (Rutten et al. 2006). Thus, trait expression may not be related to selection on a specific trait value, but rather for phenotypic plasticity, which is later molded via behavioral modulation, or modified by use, through somatic responses.

Perhaps one of the best-known examples of use-based phenotypic change is skeletal muscle. Composed of multinucleated contractile fibers known as myofibers, skeletal muscle cells are formed from the fusion of myoblasts during early embryonic development. After the initial fusion of myofibers, the total number of muscle cells typically decreases throughout life, and changes in muscle mass occur though hypertrophy of individual muscle cells, not their proliferation (Sturkie 2012). For example, adult chicken broiler strains have much heavier breast muscles than do layer strains, but these differences arise more from an increase in cell volumes and masses in the development of the former, not differences in cell differentiation and number. Likewise, increases in muscle mass in response to low temperatures or forced exercise in European Starlings elicit similar responses, with composition, performance, and masses of muscle cells changing and not absolute number (Zhang et al. 2015). However, seasonal changes in muscle mass in migratory birds are induced by photoperiod and associated increase in anabolic hormones (Ramenofsky and Németh 2014), thus, this system can be influenced by both informational and somatic responses, depending on historical selection and fitness benefits.

Both somatic and informational responses can have a temporally limited window in which they can act during development, for, as development proceeds and tissues mature in more and more functional organismal systems, opportunities for

developmental flexibility become fewer and farther between. Tissue maturation necessarily spells an end to developmental flexibility. One of the points of this review is to make it clear that this flexibility and the window for its operation is one of the properties of the developmental system that is being molded by selection. Altricial and precocial birds differ greatly in the functional breadth and temporal width of these windows of flexibility, and these also vary greatly among species under the broad altricial and precocial umbrellas. But flexibility per se, and the window for its action, may well be targets of selection every bit as important as details of tissue function and speed of growth.

c. Hormonal control of development

The development of hormones and their associated receptors in target tissues is an important component of the transition from an embryonic state to functionally mature tissues in vertebrates (Hetz et al. 2015). Endocrine signaling regulates critical biological functions including energy expenditure, development, and growth (Ricklefs and Wikelski 2002, McNabb 2007, 2006). Birds, like mammals, have two thyroid hormones, T3 (triiodothyronine) and T4 (thyroxine), which are involved in energy metabolism and thermogenesis, allowing birds to be endothermic (McNabb 2007). These thyroid hormones appear to work in concert with other components of the growth axis including growth hormone (GH) and insulin like growth factors (IGFs) to facilitate development and growth (McNabb 2007).

In precocial birds, the thyroid gland begins differentiation in the first few days of incubation, and the levels of T4 hormone begin a steady increase, faster than body mass increases. Shortly before hatch, dramatic increases in circulating T4 are stimulated by signals from the HPT axis (McNabb 2006). At a few days post-hatch,

circulating T3 and T4 hormones decrease rapidly in precocial chicks, gradually increasing again over several weeks to typical adult levels. In contrast, altricial birds show very little to no thyroid activity in the embryo. In altricial birds, beginning several days after hatch, circulating thyroid and growth hormones gradually increase to adult levels without an obvious peak throughout development (Olson et al. 1999).

Through its interactions with food availability, brood size, and energetic demands in the developmental environment, endocrine signaling modulates chick growth. For example, food restriction in young Japanese Quail resulted in suppressed circulating thyroid hormone and IGF-1 levels as well as a concomitant drop in metabolic rate (Rønning et al. 2009). Brood size reduction increases levels of IGF-1 in altricial Great Tits (*Parus major*), likely due to *per capita* increases in food provisioning from parents (Lodjak et al. 2014). Experimentally elevated levels of IGF-1 in Pied Flycatchers (*Ficedula hypoleuca*) during the post-hatch phase resulted in greater growth rate and body size, providing evidence for a potential mechanism mediating developmental rates in altricial species (Lodjak et al. 2017). The relationship between IGF-1 and somatic growth in wild birds has shown considerable promise as a mediator of phenotypic plasticity and somatic growth during early life (Lodjak et al. 2017, Lodjak et al. 2014, Lodjak et al. 2018, Dantzer and Swanson 2012). Knowledge of these mechanisms informs our understanding of how changes in phenotypic flexibility during development might evolve and the hormonal mechanisms that drive them.

d. Integrated phenotypes and hormonal pleiotropy

Hormone-mediated traits can evolve by various changes in the hormonal signaling system, including levels of hormone production, receptor affinities, and

conversion and breakdown rates, yet we still have much to learn whether these different components evolve independently or as an integrated system (Ketterson, Atwell, and McGlothlin 2009). Understanding the independence of the components, and how the individual components respond to selection is important due to hormonal pleiotropy, the situation that single hormones typically affect multiple target tissues (Pigliucci and Preston 2004, Dantzer and Swanson 2017). How tightly different tissues and organ systems (e.g., sensory, digestive, circulatory systems) are coupled together should mold the expression of different phenotypes and whether different components of the phenotype can respond to selection independently. For example, testosterone modulates a suite of different functional traits in Dark-Eyed Juncos (*Junco hyemalis*), ranging from territoriality through parental care behavior to immune system function. In this system, increased testosterone makes male juncos more likely to sire extra-pair offspring while decreasing parental effort and self-maintenance (Ketterson, Nolan Jr, and Sandell 2005, Casto, Nolan, and Ketterson 2001). The fitness benefits of elevated testosterone appear to outweigh the costs of elevated testosterone in male Dark-eyed Juncos, yet increases in nature may be constrained by negative impacts on fitness in female juncos (Ketterson, Atwell, and McGlothlin 2009).

Depending on the degree of integration, the resultant phenotype must represent a compromise in which the fitness benefits of changes in one trait outweigh the costs of those in another trait modulated by the same hormone: all trait values are not at the theoretical "optimum," and they are constrained by the correlation of tissue responses in pleiotropic effects. Weighed against the inherent sub-optimality of some trait values resulting from hormonal pleiotropy is the advantage that integrated phenotypes enjoy when rapid simultaneous adjustment of many trait values is selectively advantageous,

and these can be accomplished with very little genetic change (Ketterson, Atwell, and McGlothlin 2009).

4. Selective pressures on developmental phenotypes

a. Context-dependence

Across a range of environmental conditions, expressed phenotypes may be under different selective pressures, and those that are adaptive in one context may be maladaptive or neutral in another. For example, at low environmental temperatures, traits that maximize heat conservation, efficiency, and metabolic heat production are likely to be beneficial to endotherms (Liknes and Swanson 2011), while at high temperatures these traits would be a liability, and traits that maximize heat loss and water conservation would confer greater fitness benefits (McKechnie and Wolf 2004). However, during development, resources are often limited such that the resultant phenotype represents a compromise between resources that can be allocated to specific traits and those used to maintain the integrity of basic organismal functions needed/favored under a broader range of expected future environments (Piersma and Van Gils 2011). In this sense, observed developmental phenotypic responses may not represent optimal trait values, but may instead represent benefits in the developmental environment that outweigh potential costs in the adult/mature environment.

b. Integrated phenotypes and response to selection

Compounding the complexity of selection on the expression of specific traits, as mentioned earlier, the effects of endocrine hormones are not typically isolated to a single, targeted tissue (Ketterson, Atwell, and McGlothlin 2009, Ketterson, Nolan Jr, and Sandell 2005, Casto, Nolan, and Ketterson 2001, Dantzer and Swanson 2017)). Selection on a specific trait and the associated response may have concomitant

changes in other tissues, rather than working as independent actors under selection. There are two aspects of integration and correlation at work here. Levels of receptors, feedbacks, clearance rates, etc. can all respond in concert to selection, producing a more or less integrated hormonal response system and more or less rapid responses to selection. However, the other aspect of integration is in the overall phenotype. The hormonal systems affecting the development of different organ systems and functions can be more or less integrated across those functions. The more integrated they are, the more rapidly the overall phenotype can respond to selection. However, the more integrated these traits are, the less likely it is that phenotypes with new trait correlations will arise, that individual traits can respond independently to selection. Thus, the degree of integration, both among components of individual hormone-tissue systems and across different tissue systems, likely determines the potential for selection to shift trait values and create new kinds of phenotypes when new environmental conditions arise.

5. The relevance of developmental temperature in emergent phenotypes

a. Why focus on temperature?

Environmental temperature is central to the early development of most organisms because it influences the availability and abundance of prey, the rate of growth and development and the costs of thermoregulation (DuRant, Hopkins, Wilson, et al. 2012, Winkler, Luo, and Rakhimberdiev 2013, Andreasson, Nord, and Nilsson 2018). Environmental temperature influences the conversion of consumed resources to somatic growth through associated thermoregulatory costs (Krijgsveld, Ricklefs, and Visser 2012, Chapter 2 Dissertation) The stability of the developmental environment during early development has been considered one of the major selective advantages of internal gestation in mammals (Farmer 2000). Nearly all birds carefully time

breeding, select nest sites, and employ a variety of complex incubation strategies, which range from carefully tending mounds in Megapodes to the development of brood patches in most species to efficiently transfer heat to incubating eggs to provide a stable environment for their developing embryos (Ricklefs, Austin, and Robinson 2017) . Thus, rapid recent changes in environmental temperatures world-wide have the potential to affect the developmental trajectories and fitness of developing young birds.

Despite the considerable parental effort needed to regulate the developmental environment, both altricial and precocial offspring still experience considerable temperature variation both as embryos and as young chicks (Berntsen Henrik and Bech 2015, Hope et al. 2018). The most rapid growth occurs near optimal incubation temperatures in the range of 36-38.5°C (reviewed in (DuRant et al. 2013)) which lies within the thermoneutral zone (TNZ) of the majority, if not all adult birds (McNab 2012). Avian embryonic growth often ceases completely when egg temperatures approach physiological zero (25-26°C) (Cooper et al. 2005, Stoleson and Beissinger 1995, Arnold, Rohwer, and Armstrong 1987). Many avian embryos are tolerant of short periods of arrested development (e.g. when parents leave the nest), but embryonic growth for extended periods at suboptimal temperatures has negative effects on fitness (Olson, Vleck, and Vleck 2006). Measurements of individual egg temperatures have shown considerable between-egg variation within a nest (DuRant et al. 2016). Evidence for fitness consequences due to irregular incubation temperatures in birds extends across a wide swath of taxa, including increased costs of thermoregulation, poor body condition, and reduced immunocompetence when temperatures are outside the perceived optimum (DuRant, Hopkins, Hawley, et al. 2012, DuRant, Hopkins, Wilson, et al. 2012, Ardia, Pérez, and Clotfelter 2010).

Avian incubation and brooding is unique because offspring (embryo or young chick) thermoregulation often relies directly on heat transfer from the parents to maintain a consistent temperature for development (Deeming 2002). Regulating egg temperatures imposes a metabolic demand on the parents for thermogenesis in different environments, and, parental care acts as a mediator between ambient temperature variations and those experienced by the developing chick. In general, the effect of parental incubation behavior will be to place the developing embryo in an environment more conducive to optimal development. However, how close the incubation temperatures can be to optimal development temperatures depends on many factors, such as parental food supply, predation risk, and selection for incubation behavior related to offspring fitness (Conway and Martin 2000b, Ricklefs, Austin, and Robinson 2017).

Another unavoidable characteristic of avian attendance patterns is that eggs and young chicks cool toward ambient temperatures as soon as the attending parent leaves the nest, and developmental temperatures for birds thus have a cyclic variability stemming from parental behavior and constraints (Coe et al. 2015). Accordingly, energetic reserves guide parental thermoregulation of offspring, whether in the egg or post hatch, between foraging bouts. Thus, there is an interaction between the energetic demands of an adult providing passive heat and restoring its own energetic reserves - which includes components of their foraging strategy, the local availability of food, and its quality (Conway and Martin 2000b). Ambient temperature is another key factor that is likely to determine thermoregulation rhythms and the variation in developmental environment. Ambient temperature influences the thermoregulatory costs of incubation to the parent due to the energetic demands of thermogenesis

(Williams 1996). In addition, ambient temperature affects the rates at which eggs or young chicks cool during off-nest bouts, increasing the variability and thermal cycling of the developing young (Conway and Martin 2000a).

Finally, nest predation plays a considerable role in thermoregulatory behavior. Species that suffer from higher predation rates are less likely to make frequent trips to the nest, presumably to reduce the probability of a nearby predator detecting young (Basso and Richner 2015, Zanette et al. 2011) Because incubation requires metabolic energy and effort from parents, variation in nest temperatures both during incubation and post-hatch likely represents a tradeoff between a combination of each of these factors. These costs of accurate thermoregulation of developing embryos represent tradeoffs between offspring fitness versus parental self-maintenance and nest predation risk (Conway and Martin 2000b). The combination of more inaccessible nesting sites and greater thermoregulatory flexibility may explain why Common Swifts (*Apus apus*) faced with reduced parental food deliveries will slow down maturation and delay fledging to protect their developmental targets during their 48-65 days in the nest, whereas the ecologically similar Tree Swallow (*Tachycineta bicolor*) faced with similar food shortage will continue toward a 21-25 day fledging period despite lower fledging condition. (Shipley, unpublished data).

b. Developmental responses to temperature variations during incubation

There are many effects of variation in embryonic temperatures during incubation on development and fitness. Detrimental or suboptimal conditions during incubation are associated with shifts in multiple metrics of fitness, including increased thermoregulatory costs, reduced growth, and reduced immunocompetence (DuRant, Hopkins, Wilson, et al. 2012, DuRant, Hopkins, Hawley, et al. 2012, Ardia, Pérez, and

Clotfelter 2010). Evidence also suggests that suboptimal conditions have the potential to influence life history trajectories, ranging from decreasing life expectancies in Zebra Finch (*Taeniopgygia guttata*) incubated at low temperatures (Berntsen Henrik and Bech 2015) to skewing sex ratios in Wood Ducks (*Aix sponsa*) (DuRant et al. 2016). Body mass of developing Blue Tits (*Cyanistes caeruleus*) was positively associated with incubation temperature, but survival probability was negatively associated with higher than normal temps – suggesting that there was a tradeoff between rapid growth and survival (Nord and Nilsson 2016). These data show, across the altricial-precocial spectrum, that incubation temperatures during early development have the potential to exert considerable influence on the eventual adult phenotype and the evolution of life histories.

c. Developmental responses to temperature variations post-hatch

Compared to our knowledge of temperature effects on the phenotype during incubation, there appear to be only a few studies that have measured the effects of variation in post-hatch developmental temperatures. The nest temperatures experienced by both altricial and precocial chicks can influence growth rate and fledging success. In some altricial species, nest temperatures greater than typical appear to enhance growth or survival (McCarty and Winkler 1999, Ardia 2013, Andreasson, Nord, and Nilsson 2018) and to have a negative influence in others (Nord and Nilsson 2016, Rodriguez and Barba 2016). In Tree Swallows, high daily temperatures early in the nestling period increase growth rates (McCarty and Winkler 1999), whereas high overall temperatures throughout the entire period are associated with lower fledge rates (Ardia 2013). In precocial species, Willow Grouse chicks spend more time being brooded by their parents during low temperatures instead of feeding, resulting in slower growth (Erikstad and Spidsø 1982). Differences in the

effects of temperatures outside of the perceived thermal optimum post-hatch could be due, in part, to the characteristics of the breeding habitat itself. Warmer temperatures may be better than normal in a cold-adapted species, but detrimental in a species adapted to warmer environments. There are only a few studies that attempt to link the interaction between developmental plasticity, nestling age, and fledgling success or fitness (McCarty and Winkler 1999, Cunningham et al. 2013, Catry, Franco, and Sutherland 2011). These studies reinforce the context-dependence of performance measures and the age-dependence of thermal effects on development.

6. What do we need to know about developmental plasticity in birds?

a. Are the underlying adjustment mechanisms during development actually adaptive, and what drives them?

For highly mobile organism such as birds, determining the fitness benefits and adaptive nature of different phenotypes as adults is elusive and challenging. Many responses are assumed to be adaptive based on a general understanding of the study organism's functional biology. Thus, a real challenge with wild birds is to determine whether and precisely how variation in a given trait actually has fitness consequences. Only then can we accurately evaluate whether any plasticity behind the variation in trait values is driven by external cue-based information or is somatic and state-based (Nettle and Bateson 2015). Complicating matters, developmental responses could be the results of carry-over effects from previous selection in evolutionary or developmental time. For example, adaptive traits in early life that increase the probability of survival may still be present in adulthood when they are no longer adaptive, simply because the costs of maintaining them are minimal or selection against them is weak. Viewed in the wrong temporal window, the adaptive significance of a response during development could be missed. Similarly, when cues

that have historically contained information about future environments are no longer reliable, or vary in quality over evolutionary time, the effects on fitness may not be apparent when measured. In addition, cues that suddenly decrease in quality are likely to lead to mismatches between the historically selected phenotype and fitness in the adult environment. By documenting these effects over multiple generations and under different environments or early life events that vary in their potential to affect development we can begin to understand how developmental plasticity affects life history evolution and how different species or populations have evolved mechanisms to cope. Understanding just how various adults come to acquire the traits they do, what stresses and cues they experienced as developing chicks, and how these conditions constrain their ecological performance as adults, is probably the biggest challenge in comparative studies of the development of wild birds.

b. Are apparent developmental effects mediated by compensatory or catch-up growth, and what are the fitness consequences?

Most studies in birds have occurred over a short window during the post-hatch period, before fledging, and measurements throughout the first year of life are rare. This is largely due to the challenges of monitoring recent fledglings during this period. Is there evidence of compensatory and catch up growth in wild populations, and, if so, are there detectable costs post-fledging? For example, in many bird species, there is strong selective pressure to minimize the amount of time spent at the nest; however, reducing growth rate to cope with a period of food restriction prolongs the nestling period and increases overall predation risk. And how do tissues that are developed during periods of abnormally fast catch-up growth differ from those developed at normal growth rates? There is also evidence of negative effects of early life

malnutrition and caloric restriction in several lab-raised species; do these effects exist in wild populations?

c. How tightly integrated are the components that drive developmental plasticity?

Hormones coordinate a panoply of organismal functions, including those that regulate and guide development, growth, and maturation. Documenting and understanding how single hormones coordinate and affect multiple tissues during early life and development, and feedbacks among hormones in the developing bird, are important to understanding adaptive responses and how they are mediated and/or constrained by hormonal pleiotropy. For example, are hormonal responses tightly integrated across the entire developing organism as a unit or can selection act on a trait-by-trait, independent, basis. Studies of the integration of growth processes and their hormonal control mechanisms across the entire developing phenotype are likely to yield results of considerable importance for both applied ecologists interested in adaptation to changing climates and basic biologists interested in everything from foraging ecology to the evolution of morphology.

d. Methodological concerns

Inferences about developmental plasticity and allocation drawn solely from measures of chick mass suffer from several shortcomings. It is very likely that fitness will many times depend not on total mass but rather on the proportional size of some key body component related to fitness (e.g., fat stores, pectoral size and thermogenic capacity, etc.). Structural size, dry mass, and lipid free masses, amongst other measures, are to be preferred as they are not affected by variation in water content or proportional protein-lipid-carbohydrate composition. Future studies of comparative bird development should include assessment of trajectories of the growth of different organ

systems in addition to overall body size and mass.

7. Conclusion and Prospects

We argue that researchers could productively focus on investigating developmental effects during early life post-hatch in both altricial and precocial birds within the framework of phenotypic integration. If greater functional maturity of tissues and their physiological age limits the sensitivity to environmental constraint, then tissues at a nascent state upon hatching are prime candidates for phenotypically plastic adjustments during development. The relative immaturity of the tissues of altricial chicks at hatching suggest that they might be more flexible and potentially more responsive to environmental challenges in their post-hatch development. Of course, greater immaturity and flexibility of function also means that altricial birds may be more vulnerable to somatic, imposed restrictions on their growth, and their shorter developmental times mean that any given downturn in environmental conditions will take up a greater proportion of the chick's developmental period than it would for a precocial chick.

Developing birds are a fascinating chimera of many developing organ systems. Modern research methods have the potential to reveal a great deal about how each of these systems develop, how independently of the others, and how the details of the developmental trajectories and mechanisms in each system condition and constrain the response of each of them, and of the overall phenotype, to environmental changes. This is a fascinating area that promises us much greater understanding of the evolution of phenotypes in a group of organisms in which studies of the fitness-consequences of phenotypic variation have long been at the forefront of the research agenda.

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Chapter 2

Archetype or plastic phenotype: The effects of early life developmental conditions on thermogenic capacity

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Abstract

Our understanding of how extrinsic factors can determine phenotypes has grown immensely since the discovery of temperature-based sex determination nearly half a century ago, yet surprisingly little is known about how the developmental environment influences phenotypes in birds. In two highly precocial families of birds, Megapodidae and Anatidae, data suggest that temperature during development can have significant effects on phenotypic traits related to survival and fitness. In this study, we raised altricial Tree Swallow (*Tachycineta bicolor*) chicks for 6 days at four temperatures (31, 35, 37, and 39°C) under controlled laboratory conditions and measured the development of chick body mass, head-bill length, and primary feather growth throughout. At the end of the experiment, these measures plus basal metabolic rate

(BMR), cold-induced metabolic rate (CMR), and lipid-free organ mass were assessed. While chicks raised at 35°C (intermediate treatment) had the highest terminal body mass and 31°C the lowest, there were no differences in head-bill growth rate between any of the treatments, indicating invariant skeletal growth. Birds in the lowest two temperature treatments (31 and 35°C) also had the greatest pectoral muscle mass, which was positively correlated with thermogenic capacity as indicated by CMR. However, birds from the 31°C treatment had the slowest feather growth, suggesting a mismatch between thermogenic and insulative capacity when presented with low temperatures. These results together show that differential resource allocation between phenotypic traits can result in trait expression that may be suboptimal or even maladaptive. Our findings underscore the importance of understanding the physiological mechanisms underlying phenotypic mismatches with developmental cues under rapidly changing environmental conditions.

Introduction

For most species, the juvenile period of rapid development and maturation is perilous: at no other time during their lifespan are organisms less likely to survive. As individuals grow and mature into adults, they allocate energy and raw resources across organ systems for growth and tissue maintenance. Organisms allocate resources between growth, maintenance, and other functions in response to cues from their environment. When environmental conditions present additional demands for organisms, such as increased energetic expenditure for thermogenesis in response to low temperatures, clearly less energy is available for other functions like tissue growth. Accordingly, cues that provide reliable information about future environmental conditions should guide resource and energy allocation to different physiological systems that have provided fitness or survival benefits throughout an organism's evolutionary history (Ghalambor et al. 2007).

Developmental phenotypic plasticity enables organisms to modulate and adjust their trait values, including allocation to specific tissues, in response to cues that they experience as juveniles (West-Eberhard 2003). Plasticity allows individuals to express an array of traits from a single genotype. Expressed phenotypes can include continuous, gradual responses in traits, such as offspring growth rates (Salinas and Munch 2012) and behavior (Dingemanse et al. 2010) to discrete responses, such as sex

determination (Charnier 1965), polyphenism (Roff 1996), or induced defenses (Lampert 1993). Organisms can use cues from both their internal environment (e.g., water balance, stress response, fat reserves, etc.) and their external environment (e.g., food availability, ambient temperature, etc.). In responding to cues, organisms face a challenge in that both the internal and external environment may vary widely in their correlation between current and future conditions. This variation in cue quality, together with variation in the capacity to modulate phenotypes, should influence the degree and type of plasticity that organisms evolve (Levins 1968, Padilla and Adolph 1996). Although numerous examples of adaptive phenotypic plasticity exist (Gil et al. 2008), many instances of phenotypic plasticity have no apparent effects on fitness or survival and may even be maladaptive (Ghalambor et al. 2007, Padilla and Adolph 1996). For instance, when cues provide organisms with inaccurate information about future conditions mismatches may occur between phenotypic traits expressed during development and their fitness benefits in the future environment.

Along a range of environmental conditions, expressed phenotypes may be under different selective pressures. For example, at low environmental temperatures, traits that maximize heat conservation, efficiency, and metabolic heat production are likely to be beneficial to endotherms (Liknes and Swanson 2011), while at high temperatures these traits would be disfavored and traits that maximize heat loss and water conservation are more likely to confer benefits (McKechnie and Wolf 2004). However, during development resources are often limited such that the resultant phenotype represents a compromise between resources that can be allocated to specific traits and those used to maintain the integrity of basic organismal functions needed/favored under a broader range of expected future environments (Piersma and Van Gils 2011). In this sense, observed developmental phenotypic responses may not

represent optimal trait values, but may instead represent benefits in the developmental environment that outweigh potential costs in the adult/mature environment.

The environment that organisms experience during cellular differentiation and early development of various tissues is thought to be key to determining phenotypes. Early in life, epigenetic changes via gene promotion and repression are established (West-Eberhard 2003), alternate routes of developmental programming are set into motion (Waddington 1957), and cells undergo rapid growth and differentiation. Nearly all endotherms spend considerable effort providing a stable thermal environment for their embryos, underscoring the vital role of temperature during development.

Despite parental effort to regulate the thermal environment of embryos, young birds experience considerable variation in developmental temperatures. Most rapid growth in avian embryos occurs near optimal incubation temperatures in the range of 36-38.5°C (*reviewed in* (DuRant et al. 2013)), which lies within the thermoneutral zone (TNZ) of most, if not all, adult birds (McNab 2012). Avian embryonic growth often ceases completely when egg temperatures approach physiological zero (25-26°C) (Cooper et al. 2005, Stoleson and Beissinger 1995, Arnold, Rohwer, and Armstrong 1987). Many avian embryos are tolerant of short periods of arrested development, such as when parents leave the nest to forage, but incubation for extended periods at suboptimal temperatures resulting in reduced embryonic tissue growth rates has been suggested to have negative effects on fitness, including increased costs of thermoregulation, poor body condition, and reduced immunocompetence (DuRant, Hopkins, Hawley, et al. 2012, DuRant, Hopkins, Wilson, et al. 2012, Ardia, Pérez, and Clotfelter 2010). Because incubation requires metabolic energy and effort from parents, maintaining incubation temperatures at suboptimal ranges likely represents a tradeoff between offspring fitness, parental self-

maintenance and nest predation risk (Conway and Martin 2000).

Chicks hatch at vastly different stages of development and maturity, and accordingly, the extent to which avian parents need control the offspring environment post-hatching varies greatly (Lack 1947, Starck 1998). Altricial species hatch with most tissues at a functionally immature state: altricial chicks have poor thermoregulatory, sensory and cognitive capabilities and require considerable parental care (Starck 1998). In contrast, precocial chicks hatch with some fully functional skeletal musculature and down feathers, are alert, and begin thermoregulation shortly after hatch. Tissues that play essential roles during early stages of development (e.g., heart or digestive tract) are likely to be at similar states of maturity across the altricial-precocial spectrum, whereas tissues associated with life outside the nest (e.g., flight requires pectoralis muscles and feathers) are likely to develop later in altricial species, but typically at a much faster rate (Starck 1998). In the case of thermoregulation, the timing of active endothermy in many altricial species occurs well after hatching, after pectoral muscle and down insulation have undergone rapid growth and approached adult values. If the functional maturity of a tissue underlies its sensitivity to suboptimal or variable conditions during growth, altricial species are likely to be most sensitive to post-hatching cues (Dissertation Chapter 1). Furthermore, during their transition to active thermoregulation altricial chicks receive less parental thermoregulation, selecting altricial chicks to be especially sensitive to any developmental cues while undergoing rapid somatic growth for many tissues.

Here, we tested how environmental temperatures during the nestling period affect Tree Swallow (*Tachycineta bicolor*) developmental performance. We raised Tree Swallow chicks during their most active period of growth (days 6 to 12 post-

hatch) in a controlled lab experiment at 4 different environmental temperatures (31, 35, 37, or 39°) on an isocaloric diet. Our experimental design allowed us to quantify chick fitness for a fixed level of parental effort under different environmental conditions. We concluded our laboratory growth experiment with a series of tests measuring chick thermal efficiency (basal metabolic rate) and the ability to resist low temperatures (cold induced metabolic rate). We then euthanized the chicks to measure the effects of developmental temperature treatments on the size and development of heart, gizzard, intestine, pectoral muscle, and feathers.

Methods

Chick Rearing Experiment –

We collected 25 wild Tree Swallow chicks from 4 nests that were approximately 5-6 days old from nest boxes near Ithaca, New York, on 6 and 7 June 2016. We encouraged re-nesting by removing all chicks from each nest box to include in the experiment, rather than risk abandonment of reduced-sized broods. All research was approved under Cornell Animal Care and Use Committee protocol 2001-0051, New York State Department of Environmental Conservation Scientific Collection Permit 1477, and United States Fish and Wildlife Service Migratory Bird Scientific Collection Permit 757670.

We weighed and sorted chicks into groups of five to six individuals to receive one of four temperature treatments (31, 35, 37, and 39°C). We grouped chicks of similar starting mass to produce similar mean treatment mass and standard deviation, and randomly split up original clutches to minimize the potential for genetic or nest-specific early developmental effects. The maximum number of individuals from the same nest was 2 in a treatment. Chicks were placed individually into disposable paper

cups lined with paper towel bedding into one of four experimental incubators. We used four thermostatically controlled Little Giant Forced Air Incubators (P/N# 10300) set at 31, 35, 37, or 39° C. Humidity was maintained at 50 – 60% throughout the experiment, conducted in a Cornell animal care room.

Chicks were fed a high quality (high LCPUFA) diet that was identical in composition to that used in a previous experiment (Twining et al. 2016), based on a standard commercial Mazuri nestling feed (www.mazuri.com/mazuri/handfeedingdiets-1.aspx). The feed contains ~1.82% ALA, 3.74% EPA, and 3.44% DHA. At each feeding, all chicks were fed 6% of their expected body mass for chicks of the same age (Twining et al. 2016). This typically produced chick satiation (cessation of chick begging) by the end of each feeding. Chicks in each of the experimental groups were fed the same total quantity of feed each day of the experiment. In addition, we provided clean water ad libitum after each feeding using a wet paintbrush.

Each chick was weighed 3-4 times per day with an Ohaus Scout Pro balance (P/N# 30253019), and the daily average mass was used for calculation of growth rate. Head-bill length and primary feather growth were also measured daily using Mitutoyo Digimatic 500 calipers. Growth rates were calculated as –

$$[\ln(\text{mass or length on day } x) - \ln(\text{mass or length on day } (x-1))] / (\text{day } x - \text{day } (x-1))$$

To determine basal and cold induced metabolic rates, we used an open-flow pull-mode FoxBox respirometry setup coupled with a climate-controlled chamber at a flow rate of ~ 460 mL/min following the methods of (Lighton 2008).

We euthanized birds after measuring cold induced metabolic rate using cervical dislocation. After euthanasia, we dissected and weighed out heart, gizzard,

intestine, and pectoral muscle samples. Heart, gizzard, and intestine sample were flushed of blood or food by placing under running water and gently massaging until water ran clear or all visible food was removed (intestines). The entire pectoral muscle was excised from the left side using a scalpel. Samples were dried at 60°C for 24 hrs. Fat was extracted from each organ in a Soxhlet apparatus using 30-60 petroleum-ether as the solvent for 24 hours, and the fat-free dry mass for each part was measured after drying for 24 hrs, again at 60°C. Final mass for each of these items was determined to the nearest 0.01g using an Ohaus Scout Pro balance.

We analyzed mass, relative growth rate for mass, head-bill length, relative growth rate for head-bill, organ mass, relative organ mass, basal metabolic rate, and cold induced metabolic rate initially using linear mixed models (LMMs), and if the variance estimates for random effects were zero, then used linear models (LMs) without the random effect. In these models the random effect was nest of origin. For the models of growth rate, we used the average rate over the experiment for each individual in a LMM with temperature treatment as a categorical fixed predictor and nest of origin as a random effect. Random effects models tend to be more conservative than fixed effects, however random effects are difficult to estimate when the number of levels is < 5 (in this study, Nest levels = 4) (Bolker et al. 2009). To allow for this, we used a more conservative significance cutoff of 0.01 when interpreting mixed models.

All analyses were performed in R v3.3.3 using the packages "mgcv", data.table", "MuMIn", "knitr", and "ggplot2". All of the code and data used in the analysis is available from JRS as an annotated rMarkdown file.

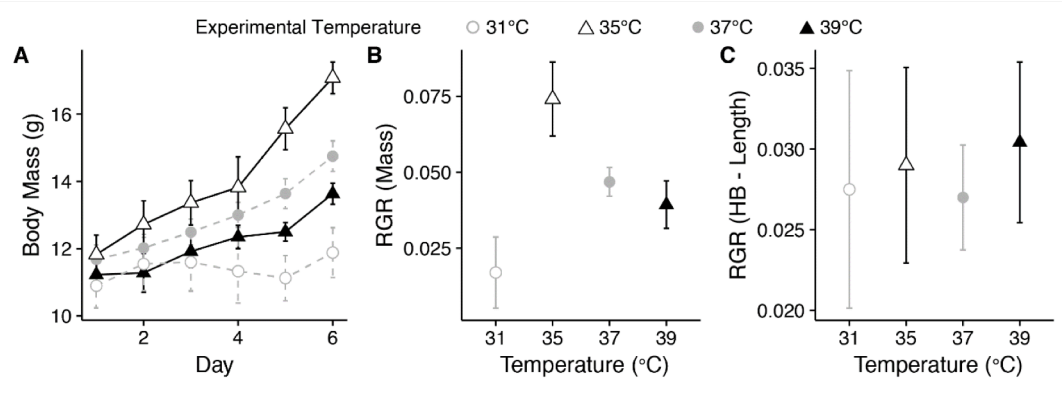

Figure 2.1 Body mass and relative growth rates from different experimental temperature treatments. Chicks were raised on isocaloric diets for 6 days during the exponential growth period at one of 4 temperature treatments: 31, 35, 37, or 39ºC.

Results

Growth Rates –

Temperature had a significant effect on the relative growth rate of chicks (LM, $F_{3,17}$ =15.00, $p < 0.001$), whereas the LMM with nest origin as a random effect did not improve fit. Coefficient estimates show that chicks exposed to the 35°C treatment had faster mass growth rates (β=0.057, $p<0.001$; Figure 2.1) than did chicks from the 31°C and 39°C treatments (β=0.017, p=0.012 and β=0.022, p=0.014, respectively). Final masses were significantly different between treatments: chicks from the 31°C treatment had the lowest final mass and chicks from the 35ºC treatment had the highest final mass (LM, $F_{3,17}$ = 18.01, $p<0.001$, β=11.8). There was no significant

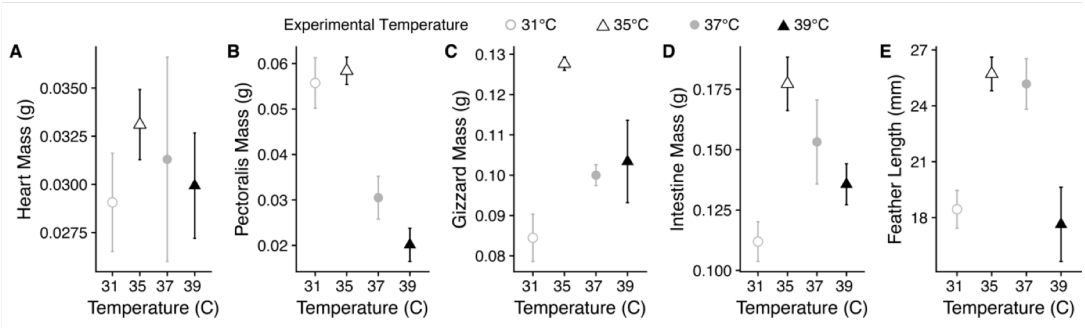

Figure 2.2. Terminal measures of heart (A), pectoral muscle (B), gizzard (C), intestine (D) mass and (E) primary feather length from the different experimental treatments. Treatment means and SE bars are shown.

difference in head-bill relative growth rate between any of the four treatments, and nest origin did not result in an improved fit in a LMM (LM, Treatment: $F_{3, 17} = 0.19$,$p = 0.9$).

Organ Masses –

There was no significant difference in heart mass between any of the four treatments (LM, $F_{3,17} = 0.64$, $p = 0.61$, Figure 2.2), and adding nests as a random effect did not significantly improve fit. Temperature had a significant effect on pectoral muscle mass (LM, $F_{3,17} = 12.9$, $p < 0.001$). Chicks in the 35°C treatment had the greatest average pectoral muscle mass, which was significantly greater than that of chicks from the 37 or 39°C treatments (Tukey, $P = 0.048$, $P = 0.008$, respectively), but not significantly greater than the pectoral muscle masses of chicks raised at 31°C (Tukey, $P = 0.99$). Temperature had a significant effect on gizzard mass (LM, $F_{3,17} = 10.9$, $p < 0.001$). Chicks from the 35°C treatment had significantly larger gizzards than did the chicks in the 31°C treatment (Tukey, $P = 0.017$), but all other pairwise comparisons of gizzard mass were non-significant. Temperature did not have a

significant effect on intestine mass (LM, $F_{3,17} = 1.67$, $p = 0.24$). Treatment had a significant effect on feather length, whereas nest origin did not (LM, $F_{3,17} = 13.9$, $p < 0.001$). Feathers from the two intermediate treatments, 35 and 37°C, were significantly longer than those in the 31 or 39°C treatments (Tukey HSD, $P < 0.05$).

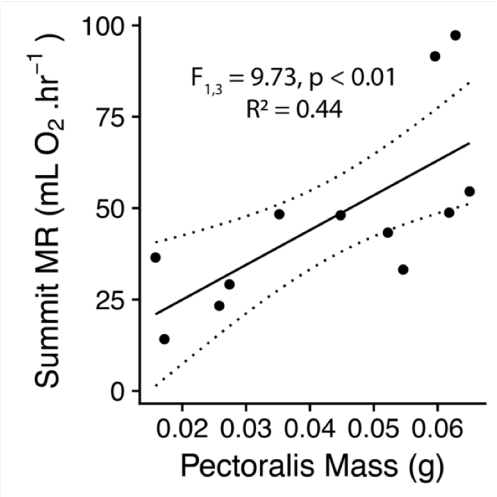

Figure 2.3. Cold-induced summit metabolic rate as a function of pectoral muscle mass.

Energetics –

Body mass had a significant effect on the whole-organism basal metabolic rate (BMR), however the effects of temperature were not significant (LM, Treatment: $F_{3,12} = 1.11$, $p = 0.39$, Mass: $F_{3,10} = 21.73$, $p < 0.001$). There were no significant differences in BMR between any of the four treatments in post hoc comparisons. There was also no significant relationship between pectoral muscle mass and BMR (LM, $F_{1,12} = 1.92$, $p = 0.19$, $R^2 = 0.072$).

Temperature had a significant effect on the whole-organism cold induced metabolic rate (CMR), but body mass did not (LM, Temperature: $F_{3,12} = 9.25$, $p = 0.003$, Mass: $F_{3,12} = 0.33$, $p = 0.57$). Including nest origin in a LMM of CMR did not significantly improve fit. Chicks from the 31°C treatment had a significantly greater CMR than did chicks from either the 35, 37, or 39°C treatments (Tukey, $P = 0.047$, $P = 0.021$, and $P = 0.011$, respectively). Although pectoral muscle mass did not affect BMR, there was a significant positive relationship between pectoral muscle mass and CMR (LM, $F_{1,12} = 12.8$, $p = 0.003$, $R^2 = 0.476$; Figure 2.3).

Discussion

For altricial birds, the nestling stage is one of the most energetically demanding and risky periods in their life: in most altricial species, nestling birds have the lowest probability of surviving to adulthood (Starck and Ricklefs 1998, Clutton-Brock 1988). Over a period of weeks, altricial species must develop from a near embryonic state to one fully capable of flight and foraging. During this period, chicks transition from thermoconformity, in which their body temperature is determined by ambient temperatures and the behavior of their parents, to full thermoregulation or actively maintaining a consistent body temperature that is generally elevated compared to their surroundings. The energetic cost of thermoregulation, which varies according to the absolute differences between temperatures inside and outside of a homeothermic organism, is one of the key factors that determines an organism's overall energy budget. As a result, the temperature of the external environment shapes and constrains the energy that is available during development for the growth and maintenance of the diverse tissues in the developing organism's body.

Here, we illustrate how resource allocation between two potentially competing functions, thermoregulation and growth, can result in phenotypic tradeoffs in rapidly developing Tree Swallow nestlings. We show that a lack of flexibility in the development of some canalized organs and structures may come at a cost under future environmental conditions because resources are always routed to these needs at the expense of others, with the potential to produce phenotypic mismatches. This conflict in functional prioritization was evidenced here in the following four ways.

Body Mass –

In our experiments, temperature during development had a strong effect on body mass: chicks from our 35°C intermediate temperature treatment grew the fastest and reached the heaviest overall mass, whereas chicks raised at 31°C were the lightest (Figure 2.1). Chicks raised at temperatures above 37 and 39°C were also significantly lighter than those in the 35°C group. 35°C lies within the thermoneutral zone (TNZ) of Tree Swallow chicks (Shipley, unpublished data), meaning that chicks developing within this temperature range did not incur additional energetic costs related to maintaining homeothermy (Burness, Ydenberg, and Hochachka 1998). Our results suggest that nestlings that develop closer to the temperature optimum put on more mass for the same amount of parental provisioning compared to chicks either above or below the TNZ, which face higher costs of thermoregulation.

Muscle Mass -

Like body mass, pectoralis mass appears to be a highly flexible trait (Fig. 2.2). The results of our experiment show a negative relationship between developmental temperature and pectoralis mass – chicks at the lowest temperature treatments had the greatest overall muscle growth. However, there was no significant difference in pectoralis mass between the two lowest treatments of 31°C and 35°C, despite individuals from these two treatments having the smallest and largest overall mass at the end of the experiment, respectively.

Skeletal growth —

In contrast to the threefold difference in relative growth rate (RGR) in mass and the differences in pectoral mass between treatments (Figure 2.1), skeletal growth RGR did not vary at all between temperature treatments. In a previous experiment on food quality and quantity effects on Tree Swallow chick development, we also found

that skeletal growth was invariant (Twining et al. 2016), suggesting that there is strong selective pressure to develop at what is likely close to the maximal skeletal growth rate. Many passerines, including Tree Swallows, face high rates of nest predation (Conway and Martin 2000) and sibling competition for food. As a consequence of these pressures in the nest, chicks are likely engaged in an arms race for high rates of skeletal growth that allow them to beg closer to the provisioning parent as well as minimize the amount of time before independence (Gil et al. 2008).

Thermogenic capacity –

While basal metabolic rates were invariant across treatments, we found a significant positive relationship between pectoralis muscle mass and cold-induced metabolic rate, a measure of thermogenic capacity (Swanson, Zhang, and King 2013). Birds rely on shivering thermogenesis from skeletal muscles, such as pectoralis muscle, as their predominant source of metabolic heat. The minimal temperature which an organism can defend against hypothermia is determined by maximal metabolic thermogenesis, which CMR is designed to estimate (Swanson 2001).

Feather Length –

Chicks raised at intermediate temperatures (35 and 37°C) grew longer feathers than those in the other treatments (31 and 39°C). One of the principal functions of feathers is to provide insulation, reducing heat loss to the environment. Our results thus illustrate a phenotypic mismatch: the 31° treatment had greater pectoralis muscle mass but shorter feathers, thus high thermogenic capacity, but poor insulation. In other words, this translates to less efficient thermoregulation for individuals raised at 31°C than the better insulated individuals raised at 35°C, despite the 31° birds having similar muscle mass and thermogenic capacity.

These four measures of developmental performance suggest a well-optimized developmental program, functioning without binding constraint in the chicks reared at 35°C, with evidence of mis-matches and trade-offs in chicks reared above and below that temperature. Chicks reared in colder temperatures would have been better off if they had been able to grow more feathers to insulate their enhanced thermogenic capacity, and those reared in higher temperatures would have benefitted from larger pectoral muscles as insurance against exposure to colder temperatures in their future.

In our experiment, we also observed phenotypically plastic responses in organ masses that are likely to have major functional implications when they covary. For example, both locomotor and digestive organs can be rapidly and reversibly adjusted in the annual cycle of Red Knots (*Calidris canutus*), driven by changing demands (Piersma and Van Gils 2011, van Gils et al. 2005). Some of these, such as the premigration enlargement of pectoralis muscle, are associated with secondary benefits like increased thermogenic capacity (Vézina et al. 2007, 2006). Tree Swallow chicks appear to attempt to prioritize allocation of resources to digestive and skeletal components during early development, which is likely due to the demands of rapid growth and the risks of predation during extended nestling periods. This is shown by the lack of differential allocation across treatment groups to heart and intestine. By ramping up these systems first, chicks develop the tools for rapidly incorporating resources that their parents provide into new somatic growth. In this sense, there is a chronological staging of organ system development that likely is driven by functionality of other systems. Future research focusing on how specific phenotypes covary with one another is necessary to provide insight into physiological constraints that may limit phenotypic and evolutionary trajectories (Buehler et al. 2012).

Together, our results show that the developmental environment can produce phenotypes that differ considerably in their capacity to meet varying demands of the environment. After hatching at a state in which they rely on their parents for thermoregulation, altricial birds typically transition to active homeothermy while they begin rapid somatic growth and grow a complement of feathers for insulation (Geiser 2008). The ability to maintain a stable body temperature in adverse conditions, or thermogenic capacity, is positively correlated with cold tolerance in birds (Swanson and Liknes 2006, Swanson 2001). For many endotherms, thermogenic capacity is positively correlated with overwinter survival, illustrating a direct link between a functional trait and long-term fitness consequences (Sears et al. 2006, Zub et al. 2014).

Through natural selection, developmental plasticity should evolve to select phenotypes that increase probability of survival and fitness under various constraints. Minimizing phenotypic mismatch between successive environments should involve ecological and environmental cues (e.g. weather, food availability, temperature, etc.) that are reliable predictors of future conditions. Information provided from unreliable cues, poor decisions, or environmental stochasticity will likely result in maladapted phenotypes, such as the chicks in this experiment reared at temperatures above optimum that performed poorly when faced with unexpected demands of thermogenesis in a cold environment.

Phenotypic plasticity in energy and resource allocation to different structures and organs during development in chicks has considerable fitness consequences. When energy, nutrients, or ingestion rates are limited, as they typically are in nature, development of all organs cannot be maximized, necessitating trade-offs. Thus, the

selective adjustment of phenotypic plasticity across environments and life stages is a critical, but elusive target. In a static environment, selection could arrive at a best trade-off among all the competing organs and structures to produce the most-fit expected phenotypes overall. However, given relentless environmental change, including changes in the correlations among cues and between cues and fitness outcomes (cf. Winkler et al. 2014), phenotypic plasticity may produce suboptimal phenotypes across a suite of environments. When cues or predictors provide information that is inaccurate or not part or a species' evolutionary history, phenotypes may be presented with environments that are very different than those that produced them. As we continue to experience an era of environmental changes at unprecedented rates, understanding the mechanisms and cues that drive developmental plasticity will be crucial for understanding the causes of variation in survival and fitness in an ever-more dynamic world.

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Chapter 3

Misread cues leads to an avian mass die off

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Abstract

A critical challenge for all organisms is using cues to determine how to allocate incoming resources according to the historical correlation between cues and fitness that drives selection for a specific response. Historically, inclement weather and the associated decline in insect activity and/or abundance has been considered one of the principal drivers of nestling mortality in insectivorous birds. This is in part due to the fact that most food resources incoming to nestlings are allocated to somatic growth and not energy storage, generally leaving few reserves when parental food deliveries run low. Here, we show that a mass mortality event in Tree Swallows, in which 71% of all individuals died over a several day period, was associated with unseasonably warm weather in the preceding weeks. This led to less resource allocation to the primary organ for generating body heat, the pectoralis muscle. This warm period was followed by a rapid change to several days of very cold weather, leading to chick hypothermia. Evidence for hypothermia, as opposed to starvation, came from three

different lines of information. None of the typical indicators of starvation were present in these chicks. Chicks from this period had similar organ and body fat to comparable chicks collected during normal weather conditions; gut contents, and all other measured organs did not differ significantly in size compared to normal years. However, the pectoralis muscles were 22% smaller in this year, and results from a laboratory experiment indicate that these small pectoral masses most likely arose as a result of the chicks growing up in warmer than normal conditions. Phenotypic responses are shaped by the historical selection in a species evolutionary history, and these results suggest that mismatches between environmental cues and the allocation to certain traits can lead to poorly adapted phenotypes and strong selective consequences.

Keywords: Phenotypic Mismatch, Hypothermia, Muscular Hypertrophy, Mass Mortality, Birds

Introduction

Phenotypic plasticity, the ability for a single genotype to express and adjust the phenotype to prevailing conditions, allows individuals to cope with both spatial and temporal variation in the environment. A critical challenge for organisms is to integrate reliable environmental cues, whether internal or from the external environment, and use this information to produce an appropriate response (West-Eberhard 2003, Winkler, Jørgensen, et al. 2014). Accordingly, natural selection should sift through the range of possible cues, and match reliable cues with their associated responses that provide the most selective benefits (Ghalambor et al. 2007). However, we know little about what happens when a historically reliable cue provides inaccurate information.

As the onset of spring is occurring earlier across much of the globe, we are witnessing a shift in animal movements at continental scales, driven primarily by an increase in mean global temperatures. In birds especially, there is a strong drive to return to the breeding grounds early each year, due in part to early arrival bringing reduced competition and greater access to resources, mates, and territories (Verhulst and Nilsson 2008b). In addition, arrival and lay date are thought to be a component of individual quality, and birds in better physical condition are able to return earlier and breed at an earlier date (Daan, Dijkstra, and Tinbergen 1990). Many species are advancing major stages in their life histories such as lay, hatch and fledge date, with some occurring 7 – 21 days earlier than a century ago, as birds adjust their timing of

reproduction to keep up with the advancing seasons (Charmantier et al. 2008, Dunn and Winkler 1999).

There is a limit however, to how early birds can return and reproduce due to the risk of adverse weather and a limited food supply. Indeed, low temperatures have been viewed as perilous for aerial insectivorous birds, as rapid drops in temperature are associated with a concomitant decrease in food availability (Winkler, Luo, and Rakhimberdiev 2013). Young chicks preferentially allocate provisioned resources to ensure rapid growth, as there is strong selective pressure for chicks to grow quickly. This is likely driven by a myriad of selective forces, including sibling competition and nest predation risk, all of which drive toward reducing the time as a flightless, vulnerable chick in most species (Conway and Martin 2000). Accordingly, when facing extended periods of low food availability due to inclement weather or low-quality habitat, young birds face a balancing act between slowing overall growth and maturity to conserve energy and reduce demand vs. minimizing the time within the nest with its associated increased food demand (Starck and Ricklefs 1998, Conway and Martin 2000).

Whereas precocial species begin to thermoregulate soon after hatching, most altricial avian species are unable to thermoregulate for the first several days to weeks after hatching. As they mature, muscles grow and begin to achieve functional maturity, which allows them to generate sufficient heat to facilitate homeothermy with the aid of the newly formed feathers (Starck 1998).

As both juveniles and adults, birds rely on shivering thermogenesis from skeletal muscle to maintain a consistent elevated body temperature, and their high

metabolic capacity has led to their ability to conquer cold environments despite their small body size (Swanson and Garland Jr 2009). The ability to maintain a stable body temperature in adverse conditions, or thermogenic capacity, is positively correlated with cold tolerance in birds (Swanson and Liknes 2006, Swanson 2001), and thermogenic capacity is positively related to pectoral muscle mass (Swanson, Zhang, and King 2013). For many endotherms, thermogenic capacity is positively correlated with overwinter survival, illustrating a direct link between a functional trait and fitness consequences (Sears et al. 2006, Zub et al. 2014). As an alternative to the demands of homeothermy, many animals can down-regulate body temperature in a process known as heterothermy, reducing their thermal gradient with their environment and thus decreasing energetic costs associated with thermoregulation (Ruf and Geiser 2015).

However, as an energy-conservation strategy, heterothermy does not eliminate the need for eventual energetic inputs, it only delays them. Starvation is ubiquitous amongst animals, and it has long been known to be a pervasive force affecting the fate of individuals and populations (Malthus 1797). Although starvation is typically viewed as 3 separate phases, it encompasses a continuous suite of physiological processes that catabolize and mobilize endogenous energetic reserves or tissues to support metabolism and basic functions of life (McCue 2010). The liver is an integral hub in starvation physiology, and in birds, besides the small intestine, it one of the most phenotypically responsive internal organs to caloric restriction (Bauchinger and McWilliams 2010). The rapid mobilization of glycogen stored in the liver is typically viewed as the earliest response to starvation, however changes in blood glucose levels due to starvation appear uncommon in birds. Instead, birds are exceptional in their ability to mobilize, distribute, and oxidize endogenous lipids rapidly (Jenni-Eiermann et al. 2002, Jenni and Jenni-Eiermann 1998, McWilliams et al. 2004). When facing

starvation, birds prioritize the conservation of proteins and first oxidize fatty acids to fuel basic functions like homeothermy.

Many insectivorous birds such as Tree Swallows breed early as a function of female quality (Winkler, Ringelman, et al. 2014, Verhulst and Nilsson 2008a, Winkler and Allen 1996, Bowlin and Winkler 2004), perceived environmental quality (Dawson 2008, Winkler and Allen 1996), or high quality resource availability (Twining and Shipley, in review), and their chicks are thought to be at risk of hypothermia or starvation during periods of unexpected low temperatures (cold snaps) as nestlings (Winkler, Luo, and Rakhimberdiev 2013, Both 2010). As global temperatures warm, many bird species are advancing their timing of reproduction, placing chicks at a greater risk of late winter inclement weather. He we explore whether mass mortality events in Tree Swallows are likely driven by limitations on energetic stores or the inability to thermoregulate leading to hypothermia and eventually death. Based on the predictions of starvation physiology, we would expect chicks during a mass mortality event to possess significantly smaller lipid reserves than healthy chicks of the same age. In addition, given the gradual development of thermogenesis ability and thermoregulation in passerine chicks (Starck 1998), we predicted that there would be age-dependent sensitivity, where chicks transitioning to homeothermy yet not possessing a full complement of feathers or mature pectoralis muscles would be most at risk.

Methods

Mass Mortality Event Data

Using the 1986-2013 Tree Swallow database, we calculated the number of chicks alive and their age for each day. We did the same for deaths, and to determine

age-dependent mortality, we divided the number of individuals that died each day of each age by the number of individuals alive each day of each age. We also calculated the total percent mortality for each day across all age groups. Individual death dates are known to occur between the last time a nest box was checked and the day the dead individual was found, which is typically every other day. From this data, we determined the severity of different mortality events based on the percentage of individuals that perished (Figure 3.1).

To determine the relationship between mortality events and weather conditions, we used daily maximum temperatures from the ASOS weather station in Ithaca, NY, and calculated rolling means for the 7 days before the mortality event. In addition, we estimated the slope of the line to determine how quickly temperature change occurred over the previous 7 days.

Organ and Growth Data

Dissected birds came from one of 3 sources. The first group (Mass Mortality) were the actual chicks that were collected after a severe mortality event on June 12th, 2016. The second group (Background Mortality) were random birds salvaged dead from the nest in 2004, 2008, or 2011, not following a cold snap event. The third group (Control) were individuals that were collected from the nest for various experiments in 2013 and 2015, and thus were euthanized and did not die of natural causes. This sampling strategy allowed us to examine differences between birds that were healthy at time of death (Control) and between individuals that died of unknown causes ("Background Mortality") during the nestling period and those that perished flowing a period of low ambient temperatures (Mass Mortality).

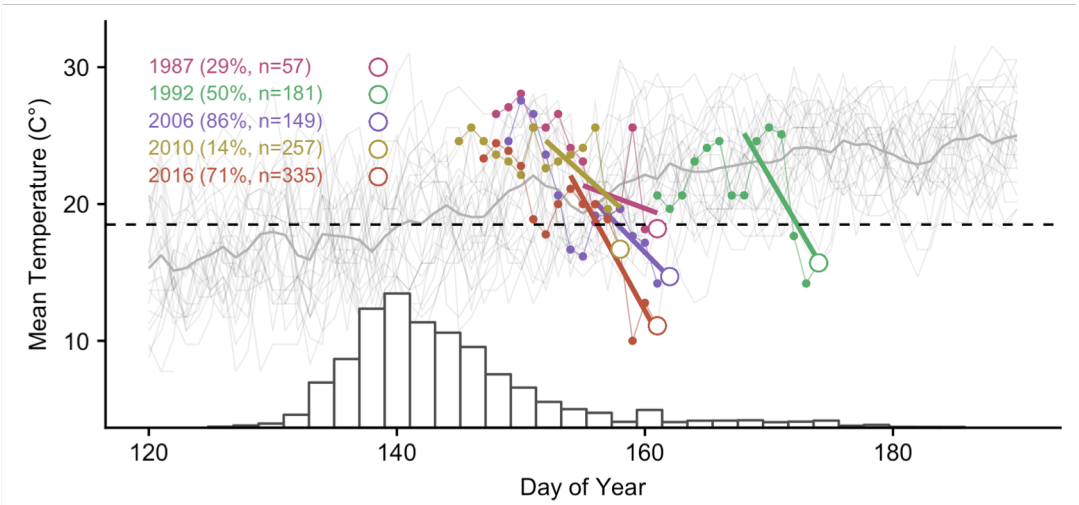

Figure 3.1. Occurrence of single day mortality events – Since 1986, there have been 5 events where more than 10% of all chicks died in a single day in Ithaca, NY. The hollow colored circles designate the date of the actual mortality event, with the colored line the change in mean temperature over the previous 7 days as the slope. Daily mean temperatures are presented for all years, with the daily average across all years as a dark grey line. The horizontal dashed line corresponds with 18.5°C, the threshold temperature for rapidly declining aerial insect abundance (Winkler, Luo, and Rakhimberdiev 2013). The bar plot is the distribution of hatch dates for Tree Swallows in Ithaca, NY from 1986 - 2015

Individual birds were dissected from each of the 3 groups (total n=79, Mass Mortality=34, Background Mortality=34, Control=11) and the pectoralis muscle from the right breast, heart, liver, stomach, intestines were excised. We also retained the carcass for further measurement of dry and lipid-free dry mass. The stomach was bisected, and the contents were removed and placed into an aluminum weighting tin. All of the organs, body, and stomach contents were placed in a drying oven at 60°C for 24 hours. The samples were then weighed on an Ohaus digital balance and the dry weight was recorded. We then performed a lipid extraction for 12 hours using a

Soxhlet apparatus containing 30-60 petroleum ether. Fat-extracted samples were then placed in the drying oven at 60°C for 24 hours, and then weighed for lipid-free dry mass.

In addition to the organ measurements, we also measured head-bill, tarsus, length of primary and contour feathers, and total mass of the bird. We determined chick age from our nest-record database to the nearest day.

Statistical analyses

We tested the relationship between the severity of the worst mortality events and climatic conditions using a generalized linear model with percent mortality as the dependent variable and minimum temperature and slope over the previous 7 days as predictors.

We analyzed the differences in dry and lipid-free dry mass of the organs and the body using a one-way analysis of variance (ANOVA) between the three treatments, with treatment as a categorical variable. We performed post hoc analyses using Tukey's Honest Significant Differences Test for multiple comparisons.

Results

Weather and Mortality Events

Rapidly decreasing temperatures (temperature slope) increased the percentage of individuals that died in mortality event as suggested by a generalized linear model (GLM, $F_{3, 7} = -4.54$, $p < 0.01$). In addition, there was an interaction between daily maximum temperature and the slope of temperature change (GLM, $F_{3,7} = 4.18$, $p <$

0.01). The opposite signs of the two variables indicate that the percentage of individual mortalities increases when the change in temperature is coupled with low maximum temperatures and is lessened when at higher maximum temperatures.

Pectoralis mass

A one-way ANOVA showed that there was a significant difference of means in lipid-free pectoralis mass between groups ($F_{2,34} = 3.8$, p = 0.032, Figure 3.3). We then conducted post-hoc comparison of means using Tukey's HSD test, the Mass Mortality group had the smallest pectoralis mass and was significantly lighter than the Control group (p = 0.036) but not the Background Mortality group (p < 0.078). There was no significant difference between the Background Mortality and the Control birds (p = 0.829).

Lipid Content Results Summary (summarizes all results below)

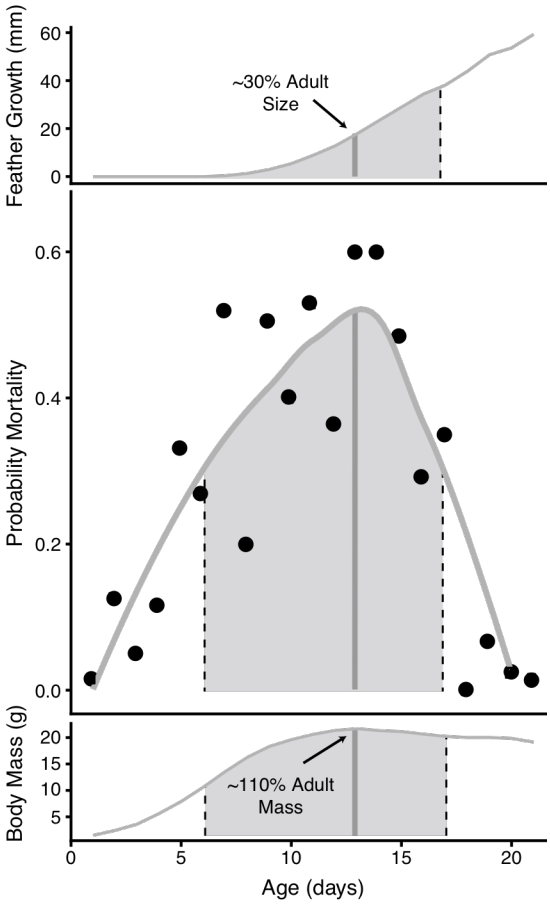

Figure 3.2: Relationship between feather growth, body mass, and mortality during 5 worst cold snaps – In the top subfigure, the peak risk of mortality during a cold snap (dark grey vertical line) coincides with the highest mass the chick (~ day 13 or 110% adult mass) will attain during development. The risk of mortality is greater than 33% (greyed areas with vertical dashed line) from day 6 to 17 in the 5 worst mortality events.

In the post-hoc comparisons, the lipid content of tissues from the Background Mortality group was significantly less than that of birds in either the Mass Mortality (3 out of 5 tissues) or the Control groups (4 out of 5 tissues). In addition, in the post-hoc comparison of summed total fat content of all organs and remaining tissues (carcass), stores of birds in the Background Mortality group were significantly smaller than those in either the Mass Mortality or Control groups ($p = 0.032$ and $p = 0.005$, respectively). There was no significant difference between the stomach lipid content in any of the groups.

Pectoralis Lipid Content

Our analysis showed that there was a significant difference of means in pectoralis lipid content between groups (ANOVA, $F_{2,34} = 34.1$, $p < 0.001$). Using Tukey's HSD test, birds from the Background Mortality group had the lowest pectoralis lipid content, and this content was significantly smaller than that in the Control group ($p < 0.001$) or the Mass Mortality group ($p = 0.008$). In addition, the lipid content of the Mass Mortality group was less than that of the Control group ($p < 0.001$).

Heart Lipid Content

There was a significant difference of means in heart lipid content between groups (ANOVA, $F_{2,34} = 8.96$, $p < 0.001$). Tukey's HSD test indicated that the heart lipid content of the Mass Mortality group was significantly smaller than that of the Control group ($p = 0.012$), and heart-lipid content of birds in the Background Mortality group was smaller than that of birds from the Control Group ($p < 0.001$); however, there wasn't a significant difference between the Mass Mortality and Background Mortality samples ($p = 0.773$).

Liver Lipid Content

There was a significant difference of means in liver lipid content between groups (ANOVA, $F_{2,34} = 4.73$, p = 0.015). In post-hoc comparisons of means using Tukey's HSD test, the liver lipid content of the Background Mortality group was significantly less than that of the Control group (p = 0.019); however, neither the Background Mortality – Mass Mortality nor the Control-Background Mortality comparisons were significant (p = 0.096 and p = 0.823 respectively).

Stomach Lipid Content

In contrast to the other organs/tissues, there was not a significant difference of means in stomach lipid content between groups ($F_{2,34} = 2.89$, p = 0.069).

Intestinal Lipid Content

There was a significant difference of means in intestinal lipid content between treatments (ANOVA, $F_{2,34} = 6.96$, p = 0.003). Tukey's HSD test indicated that the intestine lipid content of the Background Mortality group was significantly less than the Mass Mortality group (p = 0.003), however neither the Control – Mass Mortality nor the Control-Background Mortality comparisons were significant (p = 0.495 and p = 0.055 respectively).

Carcass Lipid Content

Our analysis showed there was a significant different of means in remaining carcass lipid content between treatments (ANOVA, $F_{2,34} = 5.51$, p = 0.008). We then conducted post hoc comparison of means using Tukey's HSD test, the carcass lipid content of the Background Mortality group was significantly less than the Mass

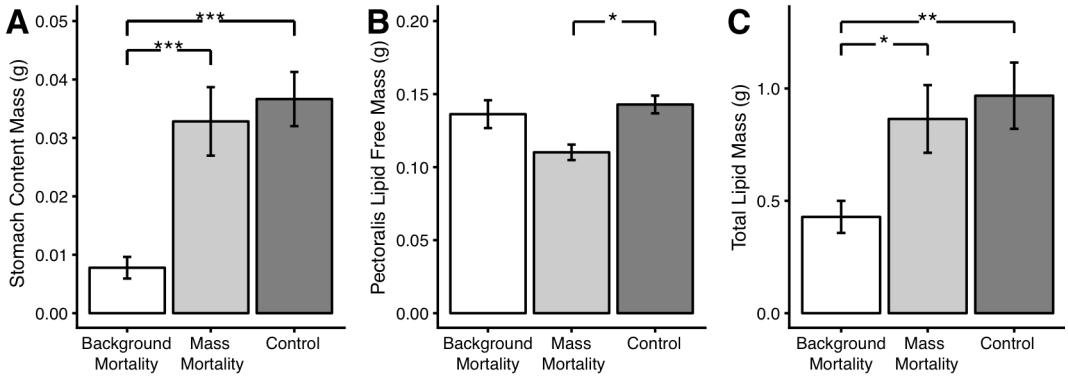

Figure 3.3. Evidence for hypothermia and not starvation – In subfigure A.) chicks that randomly died (Background Mortality) had smaller gut contents than chicks from either the 2016 mortality event (Mass Mortality) or birds sacrificed following various experiments (Control). B.) Chicks from the Mass Mortality group had significantly smaller pectoralis muscles than Control birds. In subfigure C.), the chicks in the Background Mortality group had significantly smaller total lipid mass than either treatment, and the comparison between the Mass Mortality chicks and the Control group was not significant. The results suggest that the chicks that died in 2016 Mass Mortality was likely from hypothermia and not simply starvation, as the difference in stomach contents and lipid content was not significantly different from Control birds which was greater than Background Mortality birds.
$P < 0.001$ = ***, $P < 0.01$ = **, $P < 0.05$ *

Mortality and the Control group ($p = 0.050$ and $p = 0.012$), however the Control – Mass Mortality comparison was not significant ($p = 0.867$).

Total Lipid Content (Sum of all organs and carcass)

Our analysis showed there was a significant difference of means in total lipid content between treatments (ANOVA, $F_{2,34} = 6.73$, $p = 0.004$). We then conducted post hoc

comparison of means using Tukey's HSD test, the carcass lipid content of the Background Mortality group was significantly less than the Mass Mortality and the Control group ($p = 0.032$ and $p = 0.005$), however the Control – Mass Mortality comparison was not significant ($p = 0.831$).

Dried Stomach Contents

Our analysis showed there was a significant different of means in the mass of stomach contents (ANOVA, $F_{2, 34} = 11.00$, $p < 0.001$). We then conducted post hoc comparison of means using Tukey's HSD test, the Background Mortality group had the smallest mass of dried insects in their stomach and was significantly less than the Mass Mortality group ($p = 0.004$) and the Control birds ($p < 0.001$). The was no significant difference between the Mass Mortality and the Control birds ($p = 0.790$).

Discussion

Integrating information from the environment, both internal and external, and producing an optimal phenotypic response is critical for developing organisms to maximize their potential for survival and fitness. As many birds rush to the breeding grounds each year in early spring to begin reproduction, when birds decide to breed in these environments carries with it an associated risk of unexpected weather. Accordingly, the environment the offspring experience during early development is guided by the parents previous breeding decisions and thus young birds are expected to make developmental phenotypic adjustments to make the best of the situation presented based on cues. Here, we show that in developing altricial birds such as the Tree Swallow, there is age-dependent mortality in response to rapid shifts to cooler weather, or cold snaps. In the worst of these events, we show that this mortality is likely due to the inability to maintain homeothermy during a transitory period of

development when the chick begins active thermoregulation, and not just a consequence of starvation. Finally, we suggest that the risk of mortality may be exacerbated in abnormally warm years followed by rapid drops in temperature. We reason and present evidence that developing birds may allocate resources to growing organs based on demand, and this may result in reduced performance when environments change from the conditions experienced earlier in the nestling period.

Our analysis of the worst events shows that not all individuals of all ages are affected equally, and that mortality during these aberrant events is likely context-dependent based on age. Several recent studies have shown that the effect of varying nest temperatures post-hatch have on the fledgling success, survival, or return rates of young birds depends on age – and is likely driven by costs related to size and functional maturity of organs and tissues at different stages of development (Andreasson, Nord, and Nilsson 2018, Rodriguez and Barba 2016, Cunningham et al. 2013). In our study, the youngest (< 4 days old) and oldest nestlings (> 15 days old) have the best probability of survival during severe cold snaps, while chicks around the age of 12-14 days old have the worst chance of surviving at about 40%. Interestingly, this time period (days 5 – 12) coincides with the period of most rapid growth and ~ day 13 coincides with the maximum mass chicks typically attain during development before fledging. We believe this age-dependent sensitivity is driven by a combination of factors, discussed in detail as follows.

As nestling birds grow and develop within the nest, they transition from being completely unable to thermoregulate to functioning as homeotherms. The predominant source of heat during shivering thermogenesis is the pectoralis muscle – which comprises a majority of the total skeletal muscle mass in many species. However, in

altricial species the pectoralis appears to reach adult size and functional maturity much after the chick reaches adult mass or size. In addition, growth of both primary and contour feathers also occurs relatively late in development in Tree Swallows, contributing little to insulative capacity when the chick first reaches peak mass. This could create a mismatch between thermogenic capacity and rates of heat loss from a large surface area and suboptimal insulation when the parent isn't capable of providing supplemental heat due to other constraints (self-maintenance, etc.) or the gradient between body and ambient temperature is too great. In other words, there may be a window of higher sensitivity to suboptimal conditions during development due to the conflicting demands of growth.

In this study, the cause of mortality for Tree Swallows during these spring events appear to be a combination of factors, likely driven mainly by the inability to thermoregulate and buffer against a considerable ambient temperature gradient. Our reasoning that starvation isn't the predominant cause is supported by three different pieces of evidence. First, chicks that died during the early June 2016 event did not have significantly smaller gut contents than did birds that were sacrificed for other experiments, and thus were assumed healthy at time of death. In addition, both the Mass Mortality and the Control groups had significantly larger stomach contents than did individuals dying at random non-cold-snap times (Background Mortality group). The presence of partially digested insects in the stomach suggests that parents had provisioned recently in contrast to chicks that had died of non-cold-snap causes. Second, 3 out of the 5 internal organs and the total lipid content (sum of all organs and carcass) were not significantly different between the Mass Mortality birds and the chicks that were sacrificed for experiments. There was however, lower lipid content in some organs (mainly the liver) than in birds that were sacrificed for experiments,

suggesting mobilization and use of fat for a certain amount of time before death. Finally, chicks from the 2016 mortality event had pectoralis muscles that were 22% smaller than individuals from the other groups. Skeletal muscle growth in birds responds to low temperatures with an increase in mass, much of which can be ascribed to the exercise of shivering thermogenesis. The weeks preceding the 2016 mortality event were abnormally warm for early June, suggesting there was little strain on the pectoralis muscle for shivering thermogenesis during this time. We obtained similar results in a laboratory study raising chicks at different nest temperatures, finding that chicks raised at the highest temperature allocated the least amount of resources to the growth of pectoralis muscles. If survival during periods of low temperatures is driven by the ability to thermoregulate, abnormally warm conditions followed by a period of rapid cooling suggest the possibility of a phenotypic and functional mismatch.

The adjustment of certain traits during development is likely to only be adaptive in the presence of reliable cues. In the weeks preceding the 2016 mortality event, ambient temperatures were abnormally high for early June likely leading to reduced allocation to pectoral muscle growth. When followed by a short period of abnormally cold weather, these individuals may have been exceptionally vulnerable to these conditions due to maladaptive phenotypic adjustment. Thus, it becomes apparent the correlations between cues and conditions in the evolutionary history are likely to shape the direction and magnitude of a phenotypic response during development.

Alternatively, the reaction norm or phenotypic response may not be to a specific cue in anticipation of future conditions or selected by a population's evolutionary history, but rather is a direct response to environmental conditions. Exercise induced changes have been demonstrated to occur in European Starlings

without photoperiodic cues that drive seasonal muscular hypertrophy before and during migration (Price et al. 2011). In this study, acute exercise resulted in increased levels of IGF-1 which has been shown in other studies to result in larger overall size, growth rates, and survival (Lodjak et al. 2018, Lodjak et al. 2017, Lodjak et al. 2014). This raises the possibility that pectoralis muscle growth is stimulated by environmental conditions during development and not a strategic response to a cue (Winkler, Jørgensen, et al. 2014), which would seem to increase the risk of functional mismatches during periods of high environmental variability.

Phenotypic plasticity in allocation of resources to different tissues or organs during development in chicks has considerable fitness consequences. As energy, nutrients, or ingestion rates are often limited, the development of all organs cannot be maximized, necessitating a trade-off. Thus, the selective adjustment of phenotypic plasticity across environments and life stages is a critical, but elusive target. In a static environment, selection sifts through variation in traits among all the competing organs and structures to produce an optimal phenotype. However, given relentless environmental change, including changes in the correlations among cues and between cues and fitness outcomes (Winkler, Jørgensen, et al. 2014), phenotypic plasticity may produce suboptimal phenotypes across a suite of environments.

When cues or predictors provide information that is inaccurate or not part of a species' evolutionary history, phenotypes may be presented with environments that are very different than those that produced them. As we continue to experience an era of environmental changes at unprecedented rates, understanding the mechanisms and possible relationship to cues that drive developmental plasticity will be crucial for understanding the causes of variation in survival and fitness in an ever-more dynamic world

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Chapter 4

Feather morphology allows for elaboration of insulative traits in a widespread Australian bird

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Abstract

Conflicting selection pressures can limit the expression of traits that must perform multiple functions. The contour feathers of birds play many different roles depending on species and environment, ranging from thermoregulation and aerodynamics to waterproofing or social signaling. In many bird families, including owlet-nightjars (Aegothelidae), the contour feather bears a secondary component, the aftershaft, which originates at the base and beneath the vane of the main feather shaft. Because of its placement, the aftershaft seems likely to affect only thermoregulatory function, opening the way to functional interpretation of geographic variation in this trait. First, we tested whether the aftershaft affects the insulative properties of plumage by measuring heat flux from museum specimens. Then, we examined how both aftershaft and main feather traits co-vary with environmental conditions across 5 different populations of Australian Owlet-nightjars (*Aegotheles cristatus*) inhabiting diverse habitats. Our heat flux measurements show that aftershafts provide more insulative value per unit of mass than do main feathers. Populations inhabiting cold regions had longer aftershafts with a greater barb density and a smaller main feather

shaft. Surprisingly, combined feather mass (including aftershaft and main shaft) did not differ significantly between sites, suggesting that the development of aftershafts comes at the cost of the development of main shafts. Thus, measurements of heat flux, aftershaft morphology, and the co-variation with climatic variables suggests they aid in thermoregulation and, as a result, may free the main shafts of contour feathers to be optimized for functions other than insulation.

Keywords: Feather Quality; Insulation; Feather Aftershaft; Latitudinal Variation; Nocturnal Birds; caprimulgiform; owlet-nightjar

Introduction

Natural selection sifts through variation in traits, fitting phenotypes to local selective pressures. Nearly all traits show some degree of geographic variation across a species' range, and traits often co-vary with abiotic and biotic conditions experienced by different populations (Mayr 1956). When geographically variable traits closely match environmental conditions across multiple populations, this is usually interpreted as evidence for a response to local selective pressures.

Temperature varies predictably across latitude and elevation and has the potential to drive adaptive variation in phenotypic traits (Conover and Schultz 1995). For endothermic organisms, the costs of maintaining warm body temperatures becomes ever more demanding as ambient temperatures diverge from an organism's optimal temperature (Smit and McKechnie 2010). Challenging ambient temperatures may favor changes in phenotypic traits such as plumage/pelage, body size, metabolic rate, or behavior, which should better match organisms to their environment (Piersma and Drent 2003). However, identifying traits that can respond unimodally to selection remains challenging. For example, body size generally increases with latitude (Bergmann's Rule), however many species do not show this predicted pattern, suggesting that body size may be constrained by other factors (Kooijman 1986). Weak selection, limited genetic variation or conflicting selection that favors trait characteristics in opposite directions can limit the potential for adaptive changes (Conover and Schultz 1995). This is especially the case for traits that must perform multiple functions and are exposed to diverse and/or conflicting selective pressures.

Birds have high body temperatures and metabolic rates for their size (McNab 2012), thus cold environments challenge birds to thermoregulate efficiently while

meeting their daily energetic demands. Birds can overcome some of the challenges posed by cold temperatures by increasing basal and summit metabolic rates to increase the production of heat during thermogenesis; however, this incurs an energetic tax and can force birds to spend more time foraging to meet their energy demands (Swanson and Liknes 2006). Birds can also reduce heat transfer to the environment by increasing the insulative quality of their plumage, thus reducing the overall costs of shivering thermogenesis (Stettenheim 2000). Finally, birds can employ the use of facultative hypothermia, reducing the difference between body temperature and the environment, thereby decreasing overall energetic demand (Ruf and Geiser 2015).

On the other end of the temperature spectrum, as ambient temperatures increase and approach or exceed body temperatures, conservation of heat loss to the environment becomes less significant (Wolf and Walsberg 2000). In these environments, excess body temperatures can be lethal and selection should generally favor traits that 1) minimize heat gain, 2) increase the efficiency of heat loss, and 3) reduce loss of body water to the environment (Speakman and Król 2010). Birds use a plethora of strategies to cope with hot environments, including reduced metabolic rates (Williams 2005), reduced foraging activity during the hottest part of the day (Silva et al. 2015), increased efficiency of heat loss through evaporative cooling or temporary hyperthermia (McKechnie et al. 2016) and increased lipid content of integument to reduce evaporative water loss (Williams 2005). Considering the seemingly endless varieties of feathers that exist in nature, it seems likely selection for different feather morphologies is an effective mechanism for coping with extreme environments.

Contour feathers cover most body surfaces of a bird and perform multiple functions, from maintaining an aerodynamic shape and providing insulation, to communication and signaling (Hill and McGraw 2006). Most contour feathers are composed of a central rachis flanked by a series of barbs, which are in turn flanked by a series of barbules. Feather properties, such as an increased ratio of plumulaceous to pennaceous barbs or higher barbule density, are predicted to reduce air movement and consequently the heat flux between the bird's body and the environment (Wolf and Walsberg 2000). Studies examining feather structure both within and across species suggest feather morphologies co-vary with ambient temperatures. Overwintering American Goldfinches *Spinus tristis* possess feathers with higher barb density and a greater proportion of plumulaceous to pennaceous barbs than do migrating individuals (Middleton 1986). Similar patterns between contour feather morphology and temperature have been observed in Tawny Owls *Strix aluco* (Koskenpato et al. 2016) and in the Snow/Mountain Steppe Sparrow complex, *Montifringilla sp., Pyrgilauda sp., and Onychostruthus sp*.—(Lei et al. 2002). Finally, Great Tits *Parus major* from northern populations experiencing severe winter conditions have denser feathers, but this effect is achieved by having shorter feathers with a greater density of pennaceous barbs compared to individuals at milder, southern sites (Broggi et al. 2011).

These studies illustrate three possible ways that feathers can increase their insulative capacity: (i) increase the proportion of plumulaceous to pennaceous sections of feather, (ii) adjust the number of barbs in either section of feather, and/or (iii) adjust the length of feathers. However, changing one aspect of feather morphology appears to come at the expense of other feather traits, likely due to conflicting selection and growth constraints. A fourth untested possibility exists, increasing the overall number of feathers or density per feather tract. For example, feather count varies throughout

the year in some species, but this appears to be due to loss from predation and wear (Møller 2015, Møller, Nielsen, and Erritzøe 2006, Broggi et al. 2011). However, there appears to be no clear example within a species of feather density varying adaptively in response to different environments, possibly due to the evolutionary conserved nature of feather placode development.

Another alternative for some birds to increase insulation quality of plumage is through an understudied feather component, the aftershaft. Contour feathers of many bird families possess a secondary rachis shaft known as the aftershaft, which originates from the base of the main contour feather's rachis and typically is shorter than the contour feather itself (Chandler 1916). Although some avian families lack aftershafts, the range of families that possess them is diverse, without any obvious biogeographic or taxonomic pattern (Casuariidae, Spheniscidae, Columbidae, Caprimulgidae, Trochilidae, among many others) and their function is poorly understood. The barbules on aftershafts lack hooklets and are similar in structure to downy, plumulaceous feathers, suggesting they may function to provide additional insulation (Chandler 1916), though the insulative function of variation in aftershaft structure in wild birds remains unexplored.

Here, we ask whether the aftershaft contributes to the insulative properties of plumage and study how aftershaft characteristics vary in species found in a widespread species, the Australian Owlet-nightjar (*Aegotheles cristatus*). The Australian Owlet-nightjar is found in nearly all habitats on the continent, from wet-tropical climates Cape York, to the hot xeric interior, to cold-temperate environments in Tasmania. Contour feathers in this species possess a distinct aftershaft, and, because

Table 4.1. Hypotheses of how morphological variation in contour feather traits would be related to function and the relationship to environmental predictors.

Hypotheses	**Predictor Variable**	**Expected Relationship to Predictor**
Heat Conservation	Mean Temperature of Coldest 3 Months Minimum Temperature Coldest Month	Positive relationship with length, mass, or barb density (PC1)
Heat Dissipation	Maximum Temperature of Warmest 3 Months Maximum Temperature Warmest Month	Negative relationship with length, mass, or barb density (PC1)
Resource Limitation	Annual Precipitation Growing Degree Days Rainfall Wettest ¼ Rainfall Driest ¼	Positive relationship with length, mass, or barb density (PC1)

populations do not migrate, they are subjected to vastly different ecological conditions through annual climate cycles in different regions. These characteristics make the Australian Owlet-nightjar an ideal model for the study of feather morphology in different environmental conditions. We tested the hypothesis that aftershafts increase the insulative capacity of the plumage using skin patches from museum specimens. Next, we examined structural variation in contour feathers between five populations that experience conditions ranging from wet-tropical, through arid, to cool temperate environments in light of three major hypotheses driving feather morphology, *heat conservation*, *heat dissipation*, and *resource limitation* (Table 4.1). We compared our measured eight feather traits (detailed in Methods) to environmental variables related to these hypotheses to understand selection for different traits across environmental gradients.

Methods

Field Sites

We chose five different field sites (Fig. 4.1) that span the north-south range of the species in Australia and present diverse environmental challenges. The wet-tropical site was in Queensland ("QLD", 17.4496 S, 145.3708 W), the arid site was in Northern Territory ("NTE" 23.6302 S, 132.7278 W), two warm temperate sites were in New South Wales ("NSW" 23.6302 S, 149.9717 W) and Western Australia ("WAU" 34.3093 S, 118.0635 W), and a cold temperate site was in Tasmania ("TAS" 42.0873 S, 148.2409 W).

Feather Heat Loss

To probe the functional role of aftershafts, we measured heat loss from 3 breast feather patches from a single museum specimen (CUMV 55948) with and without aftershafts.

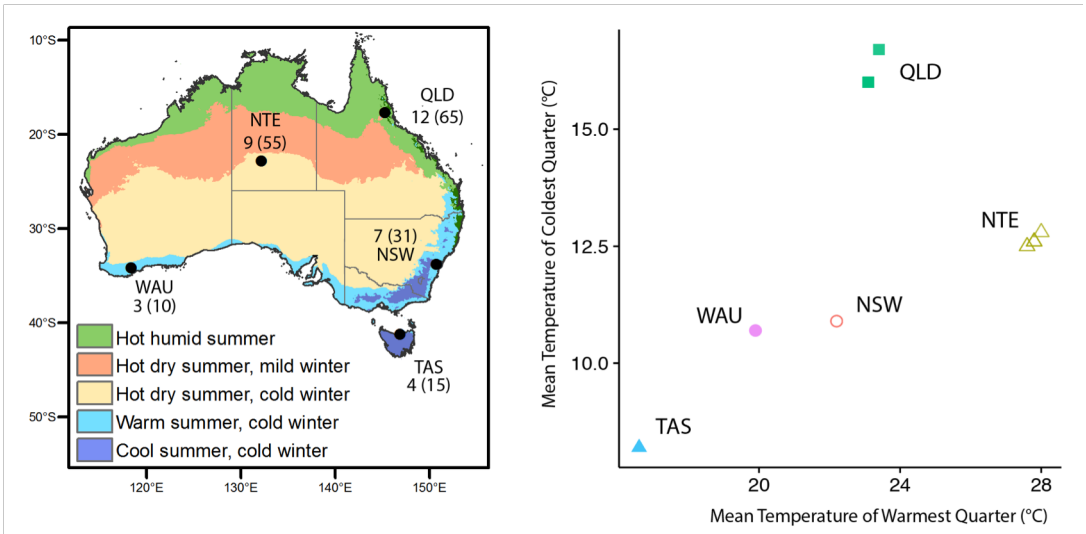

Figure 4.1. Location of field sites in Australia. Feather sampling sites span most of the climate types of Australia. Climate biomes were determined from the Australia Bureau of Meteorology data collected from 1961 – 1990 that was classified into 5 major groups. In the left subfigure, the first number below site name is the number of individuals and the second in parentheses is the total number of contour feathers. In the right subfigure, we present mean temperatures of the warmest and coldest three months for each of the sample regions. Some regions had more than one sample site (e.g. QLD and NTE), and thus have multiple points on the second figure.

We mounted skin patches to a 10x10x1mm aluminum plate, then placed skin patches in a 3D printed small wind tunnel (center section 33x16x22 cm, length:width:height) coupled with a certified, calibrated thermoelectric heat flux sensor (P/N: g-SKIN XP A-044573, greenTEG Switzerland, maximum resolution= 0.09 $W/m^2 \pm 3\%$, sensitivity= (12.74 $\mu V/W/M^2$)/C°). The wind tunnel set up consistent, repeatable air movement across the feathers, and the sensor, surrounded by the sample feather patch, measured the heat flux through the sensor from the floor of the tunnel (controlled at a temperature above ambient) to the air flow above the feathers. Wind velocity was set at 0.7 m s^{-1} using a pulse width modulated 120mm computer fan, and wind velocity was measured using a calibrated TPI hot-wire anemometer (P/N: 565, accuracy=

±5%). The sensor produces measures of flux in voltage, which was measured using a Keithley 2400 Sourcemeter. We measured heat loss from each patch of feathers in a paired experimental design. First, we measured un-manipulated patches with full length contour feathers and aftershafts; these un-manipulated patches served as a reference to measure changes in insulation after removing aftershafts and trimming contour feathers. For the first manipulation, we carefully removed all aftershafts from skin patches and re-measured heat loss to assess the effect of aftershafts on insulation. For the second manipulation, we trimmed ~5-7mm from the distal end of each contour feathers to examine the effect that feather length had on insulation. Each skin patch manipulation was measured three times to assess repeatability, and a calibration trail was run with a bare aluminum plate before each new sample. More detailed information on wind tunnel setup and methods is available in the Supplementary Materials and wind tunnel *.STL files are available from the author.

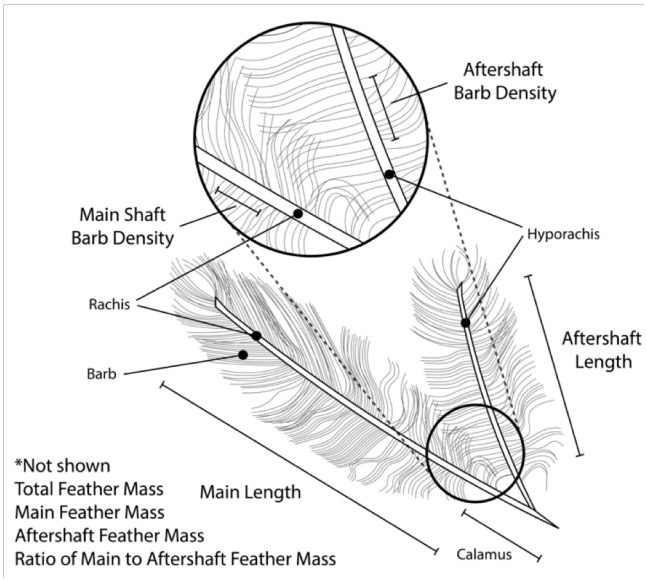

Figure 4.2. Visual description of feather variables in this study: We measured a total of 8 different feather characteristics in Owlet-nightjars. Variables that involve mass are not illustrated.

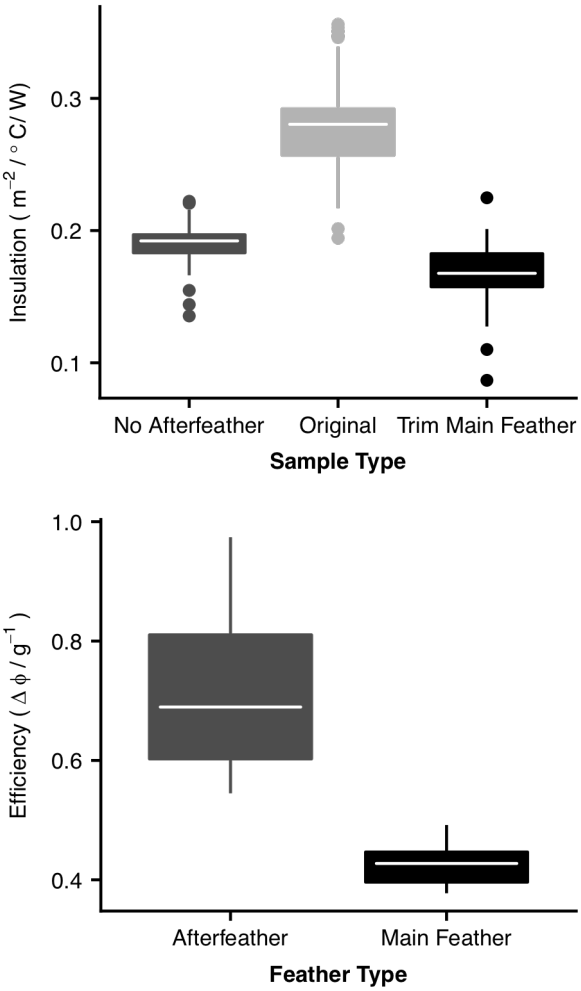

Figure 4.3. Measurements of heat flux and mass specific efficiency from feather samples: A.) Insulation (m^{-2} / °C/ W^{-1}) of the three different types of samples from the Owlet-nightjar, where the original un-manipulated samples had the greatest insulative quality. B.) Mass specific changes in heat flux, determined from dividing change in heat flux by the amount of feather material removed in our feather samples.

Feather Morphology

We obtained contour breast feathers from individuals at each of our field sites from September – October 2015 (QLD:*N*=12,*n*=65; NTE:*N*=9,*n*=55; NSW:*N*=7,*n*=31; WAU:*N*=3,*n*=10; TAS:*N*=4,*n*=15; *N*=number of individuals, *n*=number of feather samples, also see Figure 4.1). Each feather was plucked from above the upper right pectoralis muscle, approximately 1.5 cm below the furcula and 2 cm medial from the joint of the humerus. The main rachis and aftershaft of each feather were separated and placed into a labeled coin envelope until processing in the lab. We measured feather and aftershaft structure on each feather (Figure 4.2), exclusive of the calamus, using the following eight

metrics: (1) feather total mass (includes both main feather and aftershaft mass), (2) main feather mass, (3) aftershaft feather mass, (4) ratio of main to aftershaft mass (as a percentage), (5) rachis length, (6) main feather barb density, (7) aftershaft length (e.g. hyporachis) and (8) aftershaft feather barb density (cf. (Butler, Rohwer, and Speidel 2008). Barb density was measured by determining the number of barbs per 10mm length of rachis or hyporachis. Length related measurements were made by taking high resolution (6000 x 4000 pixel) images using a Nikon D7100 coupled with a Nikkor 60mm f/2.8 Micro lens (Nikon Corporation, USA). These images were processed in ImageJ (Schneider, Rasband, and Eliceiri 2012), and the length of each object was determined by drawing a line using the segmented line tool to follow the curvature of the rachis. All masses were measured using an Ohaus EX255D microbalance to the nearest 0.001 grams.

Climate Variables

We downloaded climate variables at a spatial resolution of 30 seconds of latitude and longitude (~ 900x900 meters at equator) from the BIOCLIM website at (http://www.worldclim.org/current). Data were sampled for each of the study sites using a simple script in R. From the BIOCLIM dataset, a quarter corresponds to the 3 consecutive months containing the variable of interest. For example, the warmest quarter represents the three warmest months of the year.

Statistical Analyses

We tested for the repeatability of our heat flux measurements by quantifying the intra-class correlation in a mixed effects model framework using the R package 'rptR'. Measured heat flux measurements were drift corrected according to ISO8301 calibration information provided with the sensor. After determining repeatability, we

used the average heat flux of the 3 repeated measurements in all further analyses. We tested for differences between the feather treatments using linear mixed effects models (LMMs), with treatment as a fixed effect and feather sample as a random effect.

For measures of feather morphology, there were strong correlations between different feather traits (see Results), so we performed a principal component analysis (PCA) to combine the information from the original 8 measurements into components that represent independent indices of feather traits. Each of the components derived from the PCA were tested against study site, latitude, and sample ID to identify associations with climate and geographic variables using LMMs. The LMMs were constructed with sample site and body mass as a fixed effect and feather samples nested within bird ID as random effects.

We compared the first axis of the principal component (PC1, Figure 4.4) with one of the 8 different climate variables in an LMM with feather samples nested within bird ID as random effects. We then calculated the marginal r^2 to determine how much of the variation in PC1 was associated with the climate variable. We formulated all LMMs presented in this research using the R package *lme4* with degrees of freedom estimated using Satterthwaite approximation.

Results

Heat flux measures for each skin/feather patch were highly repeatable (ICC R=0.828, SE=0.09, $p<0.001$) and the average standard deviation across all repeated measurements and temperatures was 3.99 ± 1.57 μV. Feather patches with full length contour feathers and aftershafts had the highest insulation (in (m^{-2} /C /W), $p<0.001$), while feather patches with both aftershafts removed and contour feathers trimmed had significantly lower insulation (Figure 4.3). In addition, the loss of aftershafts resulted

in a greater change in heat loss than the trimmed feathers, suggesting it has greater insulative value per unit of mass.

There was no significant relationship between feather total mass and body mass (LMM, df=20.4, t=0.53, p=0.60), but total feather mass was significantly greater in two of the populations, Northern Territory (LMM, df=29.2, t=2.08, p=0.04) and Western Australia (LMM, df=38.1, t=2.25, p=0.03).

Pearson correlation coefficients (PCC, Pearson's R) revealed strong positive correlations between the following variables: aftershaft-to-main-mass ratio and aftershaft feather mass (PCC = 0.76), feather total mass and main feather mass (PCC = 0.96), and main shaft barb density and aftershaft barb density (PCC = 0.83). To increase the independence of our measurements, we removed the variables *feather total mass*, *main feather mass*, *aftershaft feather mass*, and *main feather barb density* from the original dataset. The highest correlation in the resulting dataset was between aftershaft-to-main-mass ratio and aftershaft barb density (PCC = 0.40). However, we found the PCA results were very similar between the original and abbreviated dataset, therefore we retained all of the original variables for easier interpretability of the results presented in this study.

The PCA results showed only the first component had an eigenvalue greater than 1 (PC1 eigenvalue = 1.44). PC1 explained 37.4% of the total variance in main and aftershaft feather variables (Figure 4.4, Table 4.2). Aftershaft barb density (-0.49), aftershaft length (-0.07), aftershaft-to-main-mass ratio (-0.45), and aftershaft mass (-0.46) were negatively loaded. Thus, we interpreted PC1 as positive values indicating lower barb density with smaller proportion of aftershafts (Figure 4.4). PC2 explained

25.3 % of the total variance in feather traits. Aftershaft length (0.06) and the aftershaft-to-main-mass ratio (0.23) were positively loaded along PC2, whereas main shaft length (-0.37) and total mass (-0.62), was negatively loaded. We interpreted PC2 as positive values indicating smaller main shaft length, however there were no significant differences between populations in PC2 as there were in PC1 (Figure 4.4), and we retain PC2 here only as an axis for that figure.

PC1 varied significantly with body mass (LMM, df=17.5, t=2.14, p=0.04) and between 2 of the populations, Queensland (LMM, df=30.4, t=5.67, p<0.001) and Tasmania (LMM, df=32.4, t=-4.39, p<0.001). Population level differences in feather structure were most pronounced between the two extreme environments (wet-

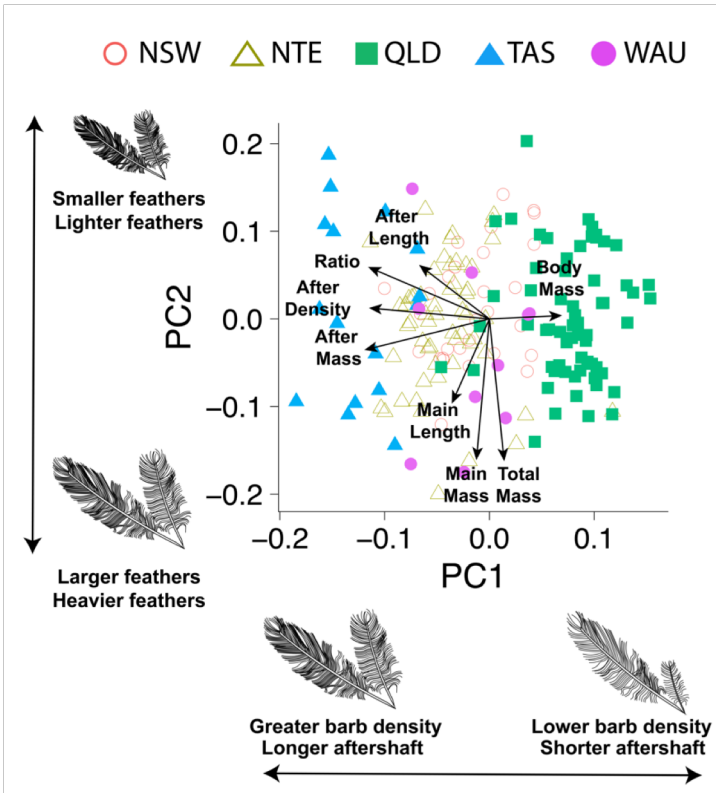

Figure 4.4. PCA of feather structure variables: High positive values on the PC1 axis correspond with less dense feathers with a smaller proportion of aftershaft. Feather samples from Queensland (QLD, squares) and Tasmania (TAS, triangles) were the most differentiated along PC1. Positive loadings on the PC2 axis correspond with smaller feathers with a lower overall mass, but do not result in significant differences

tropical – QLD (β = 1.93, se=0.34) and cool temperate – TAS (β = -2.04, se=0.46)), with consistently higher aftershaft-to-main-mass ratio, longer aftershaft length, and greater aftershaft barb density in the coldest climate (Figure 4.4). Feather structure did not differ among the warm temperate sites and arid site (NSW, WAU, and NTE) (LMM, df=33, t=0.22, p=0.82).

Table 4.2. Loadings of principal components (PC1 and PC2) used in the analysis of contour feather morphology in Australian Owlet-nightjars. Principal components were calculated using individual samples, and PC2 did not show a significant relationship with either latitude or locality.

Feather Characteristic	**PC1**	**PC2**
Eigenvalue	**1.44**	0.97
Bird Mass	**0.25**	0.01
Main Feather Mass	0.04	-0.62
Aftershaft Mass	**-0.46**	-0.13
Aftershaft Ratio	**-0.45**	0.23
Total Mass	-0.05	-0.62
Main Feather Length	-0.14	-037
Main Feather Barb Density	**-0.48**	0.00
Aftershaft Length	-0.07	0.06
Aftershaft Barb Density	**-0.49**	0.01

PC1 was correlated with 5 of the 8 measures of abiotic drivers for our hypotheses (Figure 4.5, Table 4.3). For the 2 best ranked variables, PC1 showed a positive relationship with variables related to the heat conservation hypothesis: mean temperature of the coldest three months and minimum temperature of the coldest month, with 62 and 56% of the variation explained, respectively. The next three

Table 4.3. Relationship between feather characteristics and climate variables. The mean temperature of the coldest three months of the year was the best predictor of the values seen in PC1, where low mean temperatures correspond with more dense feathers (both aftershaft and main) and the aftershaft comprising a greater percentage of main feather length. Log likelihood ($\mathcal{L}$), Akaike weight (ω_i), and the contribution of the individual fixed predictors, e.g. marginal r-squared (r^2m) are shown for each model.

Formula	$\mathcal{L}$	**AIC_c**	**ΔAIC_c**	**ω_i**	**r^2m**
Mean Temp Coldest ¼	-217	445	0	0.993	0.62
Min Temp Coldest Month	-222	455	9.97	0.007	0.56
Rainfall Wettest ¼	-228	467	22.30	0	0.48
Growing Degree Days	-229	468	23.71	0	0.45
Annual Rainfall	-235	480	34.94	0	0.32
Mean Temp Warmest ¼	-238	486	41.69	0	0.05
Rainfall Driest ¼	-238	486	41.74	0	0.05
Max Temp Warmest Month	-239	489	44.03	0	0.01

ranked variables were related to resource limitation, rainfall of the wettest three months explained 48% of the variation, whereas the number of growing degree days explained 45% and annual rainfall explained 25%. There was no evidence in our analysis supporting the heat dissipation hypothesis, as all of these predictors explained < 5% of the variation. However, the best predictors for the heat conservation (mean temperature coldest three months) and resource limitation (rainfall of wettest three months) were strongly positively correlated with each other (Pearson's R = 0.74).

Discussion

Our experimental results show that certain contour feather components are more efficient per mass at altering heat transfer and that in wild populations of Australian Owlet-nightjars; the two main feather components (main feather and aftershaft) differed across a climatic gradient. Owlet-nightjars in Tasmania, where climatic conditions are coldest, had longer aftershafts with a higher density of barbs and a higher percentage of overall feather mass was invested in the aftershaft. In contrast, owlet-nightjars from sub-tropical Queensland possessed the lowest barb density and the lowest aftershaft-to-main-mass ratio. Typically, larger feathers are thought to reduce heat loss to the environment, yet owlet-nightjars showed no significant differences in total feather mass (which includes the aftershaft and main feather) between populations. Our findings that ambient temperatures correspond more strongly with aftershaft morphology than with main feather morphology suggests that selection for increased insulative properties may operate predominantly on the aftershaft.

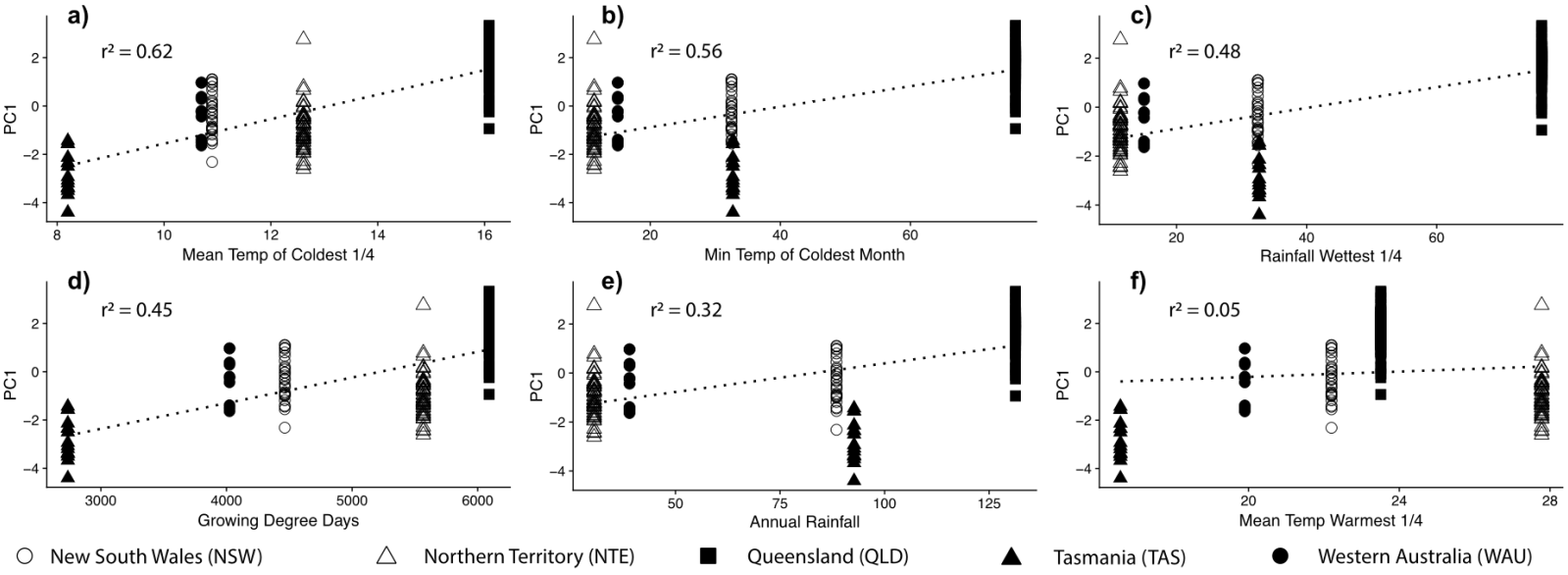

Figure 4.5. Relationship between feather traits (PC1) and a) mean temperature of the warmest three months, b) minimum temperature of the coldest month, c) rainfall of the wettest three months, d) growing degree days, e) annual rainfall, and f) mean temperature of the warmest three months. The remaining two climatic variables are not included as $r^2 < 0.05$. The two predictors most strongly correlated with PC1 (mean temp coldest three months and minimum temperature of the coldest month) are associated with the *heat conservation* hypothesis, that the costs of thermoregulation in low ambient temperatures likely drives the morphology of the aftershaft.

Patterns between environmental variables and the development of both aftershaft and main shaft morphology suggest costs and benefits to their development. Aftershafts function to increase insulation by effectively increasing the total number of feather equivalents birds have on their bodies, reducing heat loss by trapping air against the body more effectively than would single-layered feathers (Wolf and Walsberg 2000). On one hand, developing long aftershafts likely provides better insulation without compromising alternative functions of the main contour feathers. On the other, both contour feathers and aftershafts require proteins and amino acids to produce, and limitations in these resources may prevent both feather structures from achieving larger overall size.

Comparing changes in aftershaft length and the density of barbs on aftershafts suggests different components of these feathers function for different thermoregulatory roles. The number of aftershaft barbs per cm is highest in populations inhabiting cold environments and lowest in populations inhabiting the warmest site, sub-tropical Queensland (Fig. 3). This suggests that the density of barbs plays an important role in thermoregulation with higher densities reducing heat loss, consistent with other work (Broggi et al. 2011). Patterns in aftershaft length, however, appear more nuanced. Aftershaft lengths are longest in cold regions (e.g., Tasmania), shortest in regions with moderate temperatures (e.g., New South Wales), and, surprisingly, increase in length at the warmest site, Queensland (Fig 3). This slight increase in aftershaft length suggests a possibility that aftershafts may also play a role in reducing heat gain/increasing heat loss to the environment. It seems unlikely aftershafts serve to reduce cutaneous water loss in this species, as Australia Owlet-nightjars from humid Queensland had much longer aftershafts than xeric birds from the arid regions of Northern Territory.

In warm climates, especially those that are humid, selection is likely to shift towards traits that facilitate heat loss or minimize heat gain. For many species, there is a narrow range of high body temperatures that can be tolerated before excess core body temperatures become lethal and promoting heat loss is a challenge for animals that rely on evaporative cooling (McKechnie et al. 2016, Nilsson, Molokwu, and Olsson 2016, Noakes, Wolf, and McKechnie 2016). Hot humid environments could select for longer, less dense aftershafts, encouraging air movement through the feather when needed via behavioral ptiloerection, while retaining the ability to mitigate heat loss via feather overlap. In our study, birds from the warm and humid tropical habitat (Queenland) had the lowest barb density and provide some evidence for this prediction.

Patterns between environmental variables and the development of both aftershaft and main shaft morphology suggest costs, benefits, and constraints to the development of aftershafts. Our heat flux measurements illustrated that per gram of material, increasing aftershaft development provides better insulation than increases in main feather mass, likely without compromising alternative functions of the main contour feathers. Aftershafts function to increase insulation by effectively increasing the total number of feather equivalents they have on their body, reducing heat loss by trapping air against the body more effectively than would single-layered feathers (Wolf and Walsberg 2000). However, in our study increases in aftershaft size are associated with decreases in the mass of the main feather, suggesting a trade-off in the allocation of resources for generating these feathers.

Aftershafts represent a small fraction of the total mass of a contour feather, and developmental limitations within the follicle may dictate trait expression that are not necessarily driven by resource limitations. Both main feather and aftershaft are produced from the same follicle and strong correlations exist between main and aftershaft feather morphologies (in, e.g., barb density, $R^2 = 0.83$) suggests that development of some traits (e.g., the number of barbs) in these feathers is constrained by their shared follicle, possibly preventing both structures from achieving their optimal morphology. Unlike barb density, length of the aftershaft is not strongly correlated with length of the main shaft, suggesting that length may be the least constrained trait of aftershafts that must be produced by the same follicular processes that produce the main feather (Prum 2005, Prum and Brush 2002).

Regardless of the function or possible costs of producing aftershafts, these unique feathers have the potential to free contour feathers from some of the constraints that would otherwise limit adaptive evolution. Feathers perform multiple functions, from insulation and flight to sexual signaling (Hill and McGraw 2006), and this diverse set of functions may result in conflicting selection on certain feather characteristics (Butler, Rohwer, and Speidel 2008). For example, feathers that provide good thermal insulation may prove in future studies to reduce aerodynamic performance. If the development of morphologies of aftershafts and contour feathers are not prohibitively constrained by sharing a follicle, these feather types should respond to different selective pressures and diverge in morphology to better perform different functions. Our findings that owlet-nightjars inhabiting a cold site had longer aftershaft length, heavier aftershaft mass, and greater aftershaft barb counts, which should create a feather better at trapping air, are all consistent with aftershafts providing increased insulation. Some alternative hypotheses for the function of

aftershafts seem more plausible than others. For example, aftershafts are unlikely to aid in visual signals to conspecifics, as they are concealed by the main vanes of their feathers. Another possibility is that aftershafts could provide protection against feather-degrading micro-organisms. This would predict geographic patterns in the abundance or diversity of feather degrading organisms to co-vary with the development of aftershafts. Broad-scale patterns in the importance of biotic interactions, however, suggests that feather degrading organisms should be most common at our tropical, northern site, where aftershafts had the lightest mass and lowest barb density. The simplest explanation for the pattern observed in this study, both in the laboratory and in nature is that aftershafts function primarily in modifying rates of heat loss or heat gain form the environment.

Patterns between feather morphology and function are beginning to emerge. Populations or species in cold regions appear to produce shorter, denser contour feathers, which likely reduce heat loss to the environment, whereas those from consistently warm environments produce longer, less dense feathers. For birds that possess an aftershaft, these feathers likely provide additional solutions for overcoming thermoregulatory challenges without compromising main feather function. Despite the potential for specialization in feather function between aftershafts and contour feathers, developmental constraints, allocation trade-offs, and the evolutionary history of populations likely limit the elaboration of these traits. This is consistent with our findings that owlet-nightjar aftershafts show strong correlations with main feather barb densities, but weaker correlations with main feather length. Thus, despite possible developmental constraints and trade-offs between aftershaft and main shaft morphology, owlet-nightjars in cold regions produced longer, denser aftershafts compared to populations in warmer regions. This research highlights the importance

of an underappreciated aspect of feather morphology, the aftershaft. Our results suggest that future work should focus on understanding how the expression of these traits is influenced by conditions during feather growth and whether variation between populations and or species is the result of plasticity or selection for specific traits. This information will help us understand the selective pressures and evolutionary outcomes that underlie the maintenance and origin of feather trait variation.

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